



AGRICULTURAL RESEARCH INSTITUTE

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# ANNALS OF BOTANY

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AND OTHER BOTANISTS

NEW SERIES. VOLUME IV

With seven Plates, six hundred and five Figures,  
and three Diagrams in the Text

OXFORD

AT THE CLARENDON PRESS

1940

OXFORD UNIVERSITY PRESS  
AVEN HOUSE, E.C. 4  
LONDON EDINBURGH GLASGOW NEW YORK  
TORONTO MELBOURNE CAPE TOWN BOMBAY  
CALCUTTA MADRAS  
HUMPHREY MILFORD  
PUBLISHER TO THE UNIVERSITY

PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, OXFORD  
BY JOHN JOHNSON, PRINTER TO THE UNIVERSITY

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# An Investigation of the Changes in Chemical Composition and Respiration during the Ripening and Storage of Conference Pears

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With twelve Figures in the Text

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## INTRODUCTION

A CONSIDERABLE amount of work has been done on the storage of pears, but our knowledge of the chemical changes that occur in these fruits is still very limited.

A summary of the results obtained up to 1929 has been given by Emmett (1929), who investigated the chemical composition of Conference pears stored at different temperatures, with special reference to the pectic changes.

Since then Crist and Batjer (1931) have contributed a paper on the stone cells of pear fruits, and this was followed by W. W. Smith's (1935) investigation of the course of stone-cell formation in pears.

In 1936 Martin studied the distribution of sugars in Bosc pears, and in the following year he published the results of a chemical study of the ripening processes of Bosc pears (1937).

The present investigation was begun in 1936, with the idea of tracing as



fully as possible the chemical changes taking place during storage at 10° C. in Conference pears gathered at three different stages of ripeness, and of correlating these changes with the respiratory activity of the fruit.

#### SAMPLING AND STORAGE

The first gathering was made on August 4, 1936, the second on August 24, and the third on September 16. The fruit was hard and green at each gathering. It increased in size from 60.2 gm. (average fruit weight) on August 4 to 117.5 gm. on September 16. Only sound fruit of medium size was gathered. (Onslow et al 1931).

Each gathering was divided into a number of comparable samples by random selection. The method was to allot at random to every fruit a number and then to employ an arrangement for drawing these numbers at random. The time occupied between gathering and the completion of sampling was about twenty-four hours.

A certain number of samples, called the initial samples, were frozen to -20° C. immediately after sampling was completed. The rest were placed on wooden trays in a storage cabinet<sup>1</sup> at 10° C. and removed thereafter one by one on a series of predetermined dates. Each sample was, on removal, frozen to -20° C.

All samples were frozen to -20° C. and stored thereafter at that temperature in air-tight tins. Before being placed in the tins the pears were cut in half and the seeds were removed. The peel was not removed. In the preparation for chemical analysis a sample was ground to a fine powder in the frozen state. This powder was then systematically mixed and weighed out into aliquot lots of 100 gm. and then put aside again at -20° C. in covered glazed earthenware pots.

As the results were to be expressed in terms of unit original fresh weight, all samples were weighed (1) at the outset, (2) after removal from storage, and (3) before grinding. When rotting had occurred in storage and rotten fruits or parts of fruits were discarded before freezing, the sample was weighed before and after the removal of the rotted fruits. Tables V, VI, and VII summarize all results obtained.

#### METHODS OF ANALYSIS

##### *Nitrogen estimations.*

Total nitrogen is estimated on 50 gm. of the frozen tissue by the Kjeldahl method.

In order to obtain the insoluble nitrogen, 100 gm. frozen tissue is allowed to stand overnight at 10-15° C., then washed into a Soxhlet thimble, with

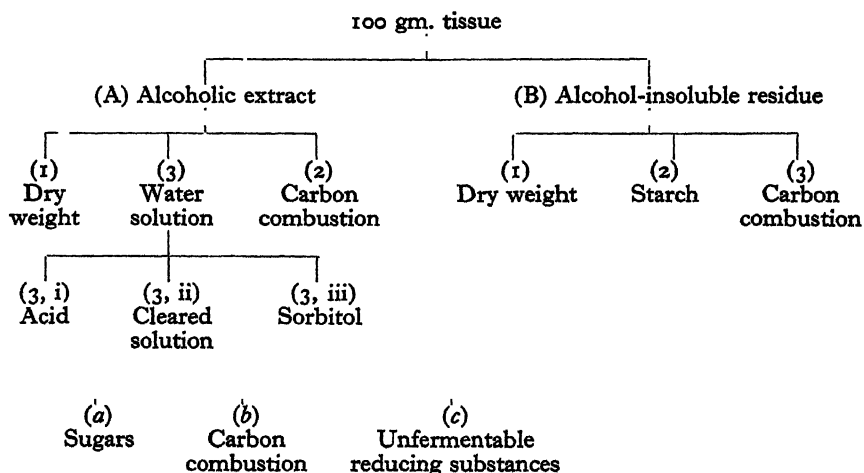
<sup>1</sup> The storage cabinet was a gas-tight metal box of 140-litre capacity through which fresh air from outside the building was drawn, at a rate of 70 litres an hour. This air, before entering the cabinet, was scrubbed free from carbon dioxide by soda lime and then humidified to 95 per cent. relative humidity in a calcium chloride tower.

85 per cent. alcohol. The thimble and contents are then extracted with 200 c.c. of 85 per cent. alcohol in a vacuum extracting apparatus, keeping the temperature of the bath at 50° C. for four and a half hours.

The tissue is dried in the thimble in an oven at 85° C., then transferred to a Kjeldahl flask and N estimated. The alcohol-insoluble nitrogen is referred to as protein nitrogen.

#### Other estimations.

Samples of the frozen powder were extracted with hot 80 per cent. alcohol for four hours and estimations made according to the following scheme.



A. The alcoholic extract was made up to a litre with alcohol and aliquots taken for the following:

(1) *Dry weight.* 10 c.c. were used for dry weight determinations in a flat-stoppered weighing dish and dried for 48 hours in air at 50° C.

(2) *Carbon of alcoholic extract.* 5 c.c. were used for the carbon combustion, using the wet method described by Raistrick (1930) with the modification of apparatus employed by Dr. E. J. Maskell of the Botany School, Cambridge. The alcohol was removed by warming on a water-bath at 30° C. under reduced pressure, and the last traces of vapour by a slow stream of air.

(3) 800 c.c. of the alcoholic extract were evaporated under reduced pressure at a temperature of approximately 30° C. and the residue made up to 200 c.c. with water.

(i) *Acid.* Aliquots were titrated with 0.1 N/NaOH using phenolphthalein as indicator, and the titratable acidity was calculated as malic acid.

(ii) 100 c.c. of the aqueous solution were neutralized and cleared with basic lead acetate, filtered, and de-leaded with sodium phosphate, made up to a suitable volume, filtered through a dry paper, and used for sugar estimations.

(iii) *Sorbitol*. The estimation of sorbitol was carried out in only a few cases, when the following method, which gives approximate results, was used. The dibenzal derivative of sorbitol was prepared according to the method of Werder (1929), which has been elaborated by Bleyer, Diemair and Lix (1931) and by Martin (1937). The isolation and identification of sorbitol are described in a subsequent section.

50 c.c. of the water solution were warmed with 5 gm. of Merck's medicinal charcoal and then filtered. The filtrate was fermented with yeast to remove the sugars present. When fermentation was complete, alumina cream was added, and the liquid was filtered. The clear colourless filtrate thus obtained was made up to 200 c.c. Aliquots of this solution were taken so that the yield of the dibenzal derivative was approximately between 0.150 to 0.450 gm. 0.1 gm. of sorbitol is equivalent to 0.1968 gm. of dibenzal derivative. The solution was evaporated to dryness, 1 c.c. of 50 per cent. sulphuric acid and 0.5 c.c. of benzaldehyde were added and well shaken for 5 to 15 minutes and then left at 1° C. overnight. 50 c.c. of water were then added and left to stand at 1° C. for several hours, after which the solution was made alkaline with a slight excess of anhydrous sodium carbonate, and left at 1° C. for one hour longer. The solution was then boiled for 2 to 3 minutes and cooled. The precipitate was broken up with a glass rod and filtered through a weighed glass crucible, washed with water, and dried at 100° C. to constant weight (about 12 hours).

(a) *Sugars*. Reducing sugars were estimated before and after hydrolysis by Maskell's modification of Shaffer and Hartman's (1921) micro-method. Hydrolysis was effected by heating at 60° C. for 25 minutes in 0.5 N HCl.

Fructose was estimated directly after oxidation of the glucose with 0.2 N alkaline iodine solution at 10° C. and removal of excess iodine with sodium sulphite. The difference between the reducing value before hydrolysis and that for fructose represents glucose.

(b) *Carbon in cleared solution*. Aliquots of 5 to 10 c.c. of the cleared solution were used for the estimation of the carbon content, using the same method as in A. 2, allowance being made for the carbon content of the lead acetate used in clearing.

(c) *Unfermentable reducing substances*. Aliquots of the cleared solution were fermented with yeast in order to determine whether there was any quantity of unfermentable reducing substances. This was only carried out in a few cases, and the amounts found were very small.

B. The alcohol insoluble residue was treated as follows:

(1) *Dry weights* were obtained by drying the residue at 100° C. for 24 hours.

(2) *Starch*. A weighed amount of the dried alcohol insoluble residue was shaken up with 1 per cent. potassium oxalate solution for 5 hours (Widdowson (1932), washed with water until free from oxalate, and then hydrolysed with taka-diastase. The reducing value was then obtained by the method given in 3 (a), and related to the reducing power of a pure hydrolysed apple

starch. The value obtained for starch by the above method is used in all the calculations and in plotting curves.

Starch was also estimated in the alcohol-insoluble residue with taka-diastase, without previous extraction with 1 per cent. potassium oxalate solution. This method gave higher values for starch than those obtained when the residue was first extracted with potassium oxalate solution. The difference in the starch values for the two methods remained approximately constant throughout the storage. There is thus a definite fraction which is extracted by potassium oxalate and which is hydrolysed by taka-diastase with production of reducing sugars.

(3) *Carbon of alcohol-insoluble residue.* A weighed amount of the alcohol-insoluble residue was estimated for carbon content, using the method given in A 2.

Other fractions which may be called 'derived fractions' and which are alluded to in this paper are herewith explained.

*Total glucose* is the sum of free glucose, half the sucrose, and starch calculated as glucose.

*Total fructose* is the sum of free fructose and half the sucrose.

*The 'non-acid' fraction of the lead acetate precipitate* brought down on clearing is that portion of the lead acetate precipitate which is left after deduction of the malic acid. It can be estimated in terms of carbon from the difference between the carbon of the alcoholic extract and the carbon of the cleared solution.

*Residue less starch* is the alcohol-insoluble residue minus starch, as determined by taka-diastase after extraction with potassium oxalate.

*The unestimated fraction.* This term has previously been used to connote the fractions obtained by deducting the sum of the sugars and malic acid estimated by analysis from the dry weight of the alcoholic extract. Its use is retained in this sense, although it contains the sorbitol, because it is not yet certain how precise is the estimation of sorbitol. It appears that in the present case sorbitol accounts for about half the unestimated fraction.

## METHODS OF ESTIMATING THE PRODUCTION OF CARBON DIOXIDE AND OF VOLATILE SUBSTANCES

### *Carbon loss by respiration.*

Two methods of measuring the carbon dioxide given off by the pears were used.

In the first method the respiratory activity of groups of 6 individual fruits was determined by the Pettenkofer tube method. These 6 pears were replaced, at intervals, by 6 others from a sample which was kept in store at 10° C. In this way, a mean respiration curve of a complete sample of 30 pears was obtained.

In the second method the amount of carbon dioxide produced from all the pears in the storage cabinet was measured by means of the divided tube apparatus, which has been described by Kidd, West and Gane (1935). The air current from the cabinet was passed through the differential resistance and the carbon dioxide in a known fraction of it was measured by means of Pettenkofer tubes containing baryta. The ratio between the divided streams of air was checked at frequent intervals by passing a measured amount of carbon dioxide through the apparatus. The values given in the tables of results in the column headed 'Carbon loss by respiration' are those obtained by this second method.

#### *Volatile substances.*

The carbon-containing substances, other than carbon dioxide, produced during respiration were estimated by passing CO<sub>2</sub>-free air over samples of 15 fruits, then through a soda-lime tube to absorb carbon dioxide, and finally through a furnace containing CuO, the resultant carbon dioxide being measured in a Pettenkofer tube containing baryta. These samples were kept in darkened glass desiccators, and the rate of ventilation per fruit was the same as in the case of the main lot of storage samples.

#### *Alcohol and acetaldehyde.*

Alcohol and acetaldehyde contained in the pear were estimated together by the steam distillation of 100-gm. samples of the frozen, ground tissue.

The distillate was oxidized with potassium dichromate and sulphuric acid, the acetic acid distilled over being estimated with standard sodium hydroxide solution (Trout, 1930).

### THE ISOLATION AND IDENTIFICATION OF SORBITOL

From the material of the first gathering several hundred grams were treated as in the method of estimation up to the point of obtaining the clear colourless filtrate after fermentation and clearing with alumina cream. The filtrate was then concentrated under reduced pressure to a thick syrup.

This syrup, presumed to contain sorbitol, was used for the preparation of the insoluble benzal compound (Davis et al, 1926; Haas and Hill, 1932; Tutin, 1925). An equal weight of 50 per cent. sulphuric acid was added to the syrup, followed by the addition of slightly less benzaldehyde, the mixture being well shaken and left to stand overnight in the cold. The white precipitate which had formed was filtered off, washed with water, and dried. To regenerate sorbitol from the benzal derivative the compound was hydrolysed by boiling with 5 per cent. sulphuric acid until solution was complete, when the excess benzaldehyde was removed by steam distillation and the excess sulphuric acid with baryta. The filtrate was concentrated to a syrup, treated with hot 95 per cent. alcohol, and left to crystallize, when colourless needle-shaped crystals were formed, which gave a M.P. of 91° C. All the samples

of sorbitol which were obtained from the Conference pears gave a melting-point of  $91^{\circ}$  to  $93^{\circ}$  C., after being dried *in vacuo* over  $P_2O_5$  at room temperature and also in a vacuum oven over  $P_2O_5$  by gradually raising the temperature to  $90^{\circ}$  C. Davis, Slater and Smith (1926) state that the melting-point determinations of sorbitol are of no value unless the moisture content can be stabilized. The literature on the subject of the melting-point of sorbitol is very conflicting (Boussingault, 1872; Fischer, 1890; Hitzemann and Tollens, 1889; Lippmann, 1927; Nanji and Paton, 1924; Rüber et al, 1925; Strain, 1934 and 1937; Vincent and Delachanal, 1889).

Owing to the unreliability of the melting-point as a means of identification of sorbitol, it was decided that further evidence must be obtained in order to identify the substance isolated.

With this end in view, hexa-acetyl sorbitol was prepared from the sorbitol obtained from the benzal compound, by heating with acetic anhydride in the presence of a few drops of pyridene, and from this solution the acetyl derivative was separated in two ways:

1. By careful dilution with water, when colourless prismatic crystals separated, which gave a melting-point of  $98^{\circ}$  to  $99^{\circ}$  C.
2. By extracting the diluted solution several times with ether, when pale yellow prismatic crystals were obtained, which were purified by recrystallizing several times from ethyl acetate, and which gave a melting-point of  $98^{\circ}$  to  $99^{\circ}$  C.

As further proof that the hexa-acetyl derivative of sorbitol had been formed, the compound was saponified with alcoholic potash and the excess potash titrated with N/1 hydrochloric acid, when the theoretical result for hexa-acetyl sorbitol was obtained. A mixed melting-point was also taken of the hexa-acetyl sorbitol obtained from the pears and that prepared from Kahlbaum's pure sorbitol when a melting-point of  $98$  to  $99^{\circ}$  C. was obtained.

The hexa-acetyl compound formed from the pear sorbitol was then hydrolysed by heating with 5 per cent. sulphuric acid, the acetic acid got rid of by steam distillation and the sulphuric acid with baryta and the filtrate evaporated to dryness under reduced pressure. The syrup obtained in this way was then dissolved in hot absolute alcohol and left to crystallize, when colourless crystals were obtained which gave a melting-point of  $91^{\circ}$  C.

#### DRIFTS OF RESPIRATORY ACTIVITY

The records of respiratory activity (c.c. carbon dioxide per 10 K. H.) are set out in Figs. 1, 2, and 3. The results obtained by the two different methods described above agree reasonably up to the period of the climacteric rise. Over this period the activity of the bulked group, i.e. all fruits of the chemical samples held together in the storage cabinet, rises more quickly than the average activity of a group of individuals each isolated from one another in separate respiratory containers. Kidd and West have described this effect in the case of apples (Kidd and West, 1933) and have traced its origin to the

stimulating effect of ethylene produced during the climacteric rise. Where fruit in bulk is used the first individuals to reach this climacteric will automatically tend to stimulate the onset of the climacteric in the other fruits. There is also a suggestion that the respiratory activity of the bulked samples in the post-climacteric phase is higher than that of the isolated individuals.

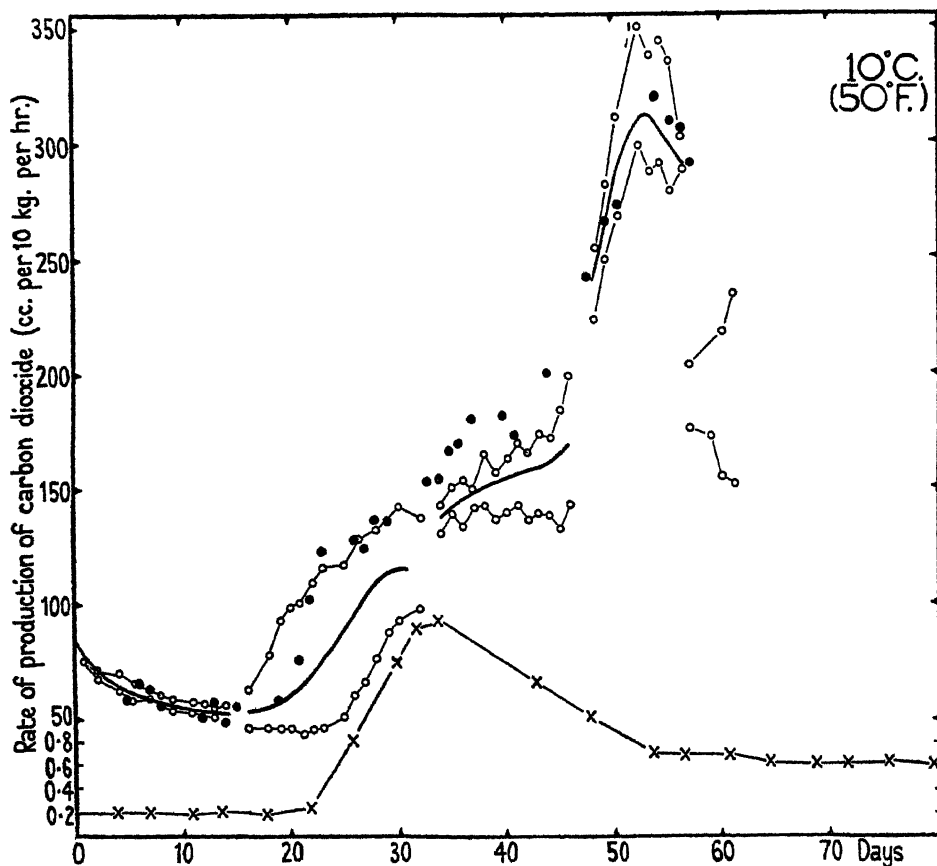


FIG. 1. Carbon dioxide-production and evolution of volatile substances during storage (first gathering). Scale for rate of production of carbon dioxide from combustion of volatile substances is magnified fifty times as compared with that for respiratory activity.

*First gathering.* In the case of the first gathering (Fig. 1) the average of the six individuals (heavy continuous line) agrees closely up to the 20th day with the results obtained by the split current method on the fruit stored in bulk (heavy solid dots not joined up). Between the 20th and 30th day the activity of the latter rises steeply, the slope of the rise being as steep or steeper than that for any individual fruit. The extremes of individual behaviour are plotted as open circles joined up. Of the individuals during this period one had already begun to rise by the 6th day and one did not begin to rise till the

25th day. Consequently the average activity of the group of individuals rises more slowly than that of any one individual. The records of the individuals show that the initial rise in activity from about 50 c.c. to about

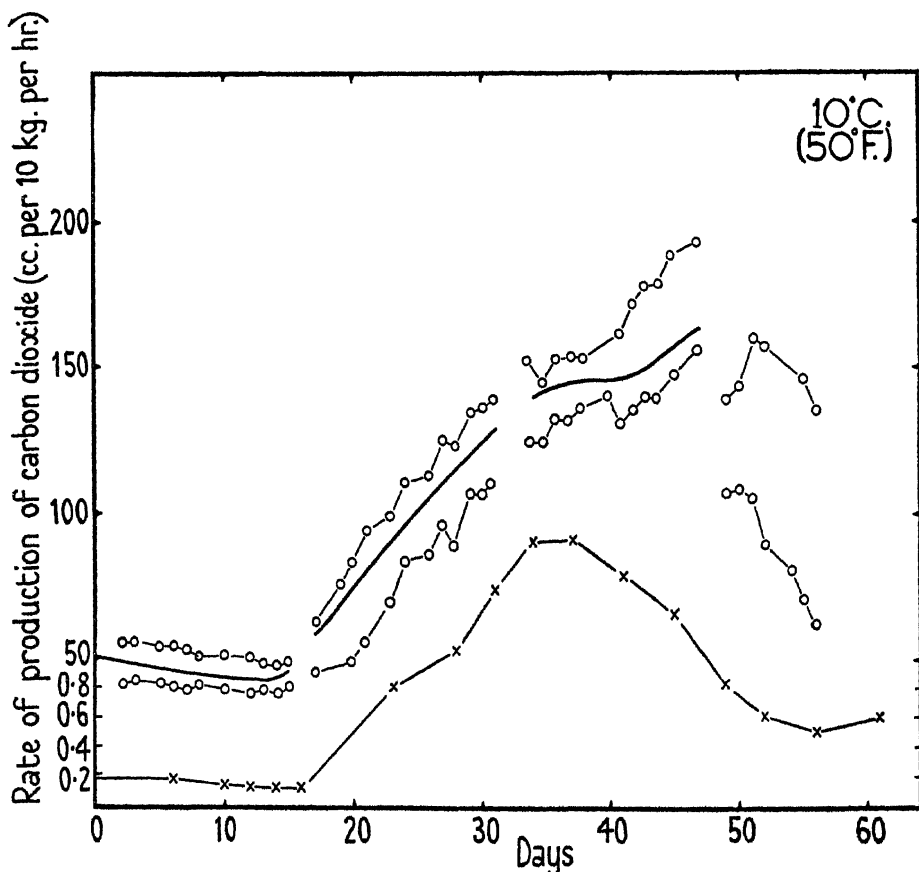


FIG. 2. Carbon dioxide-production and evolution of volatile substances during storage (second gathering). Scale for rate of production of carbon dioxide from combustion of volatile substances is magnified fifty times as compared with that for respiratory activity.

125 c.c. was followed by a period of steady activity. The dimensions of this initial rise are similar to those of the climacteric rise in respiratory activity in apples.

On the 33rd day the third sub-sample of six fruits was removed from the bulked group in the storage cabinet and the respiratory activity of the individual fruits was measured. It is interesting to note that their average respiratory activity thereafter appears less than that of the fruit remaining in bulk. Of the six individuals, two exhibited a steady rate of activity during the period they were under observation, while four showed the beginning of a further rise in activity.

From the 45th day onward the fruit of the first gathering began to become



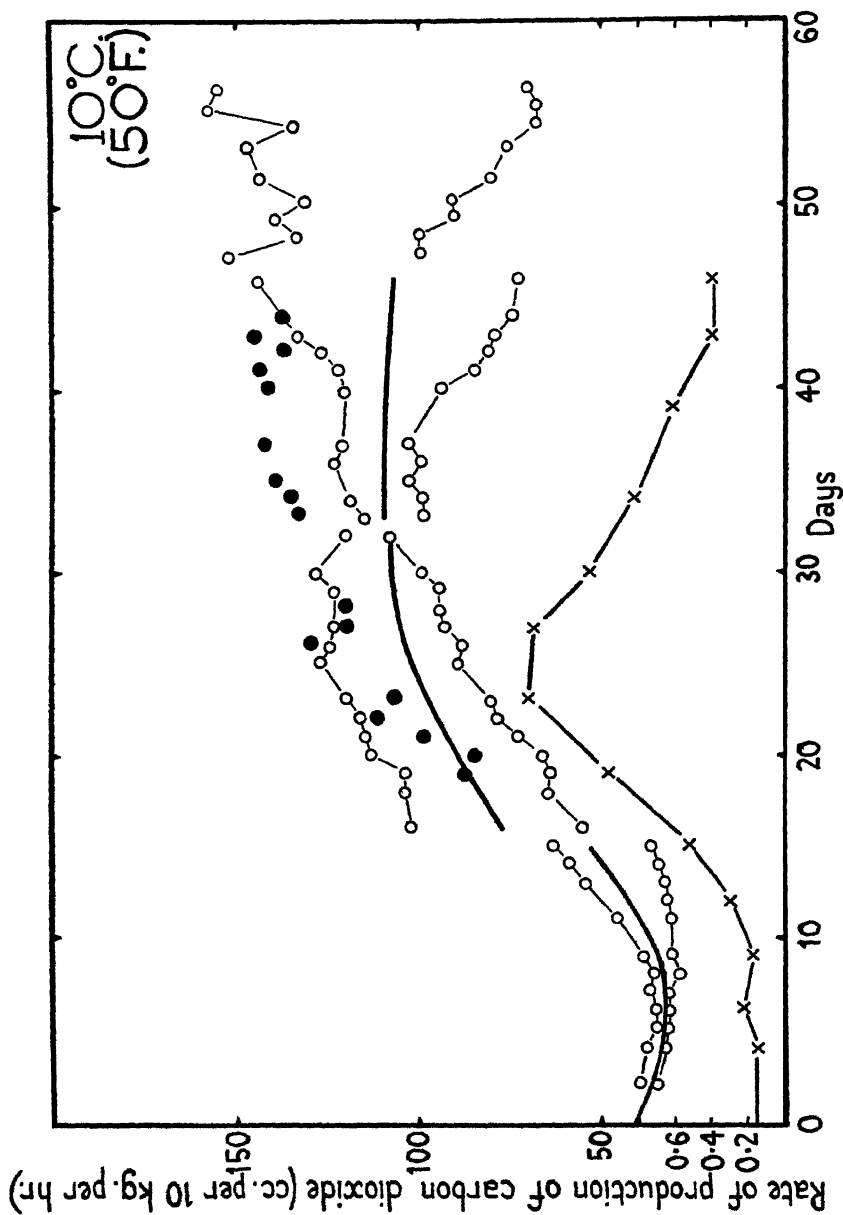


FIG. 3. Carbon dioxide-production and evolution of volatile substances during storage (third gathering). Scale for rate of production of carbon dioxide from combustion of volatile substances is magnified fifty times as compared with that of respiratory activity.

meally<sup>1</sup> and to develop rots. It never attained a melting, juicy consistency. The respiratory activity of the bulked group and of the individuals of subsample 4, removed from the bulk-group on the 48th day, rose to a sharp and high maximum and rapidly fell. In the absence of rots the indication was that this fall would be to zero activity. A relatively sudden rise to a high maximum of activity falling afterwards rapidly to zero is the type of behaviour associated with the breakdown and browning of apples that occur in storage at low temperatures (Kidd and West, 1925-6).

The record of the rate of production of carbon, other than that of carbon dioxide, is steady at a very low level up to about the 20th day. This record is plotted as crosses joined up. It rises to a maximum on about the 33rd day. It then declines steadily. The maximum rate of production of volatiles thus corresponds to the maximum of the initial rise in respiratory activity. The final burst of activity associated with development of mealiness has not a counterpart in activity of volatile-production.

The measurement of the carbon from volatiles was continued for eighty days, until all the fruits had become 'sleepy' and 13 out of 15 were partly or completely rotted. The decline in activity of volatile production continued up to about the 50th day and was thereafter constant at a value about three times that given by the fruit immediately after gathering.

It is known that many fruits contain traces of ethyl alcohol and acetaldehyde as normal constituents of the tissues, and that in the post-climacteric phase these substances tend to increase in amount. In the present case determinations were made at intervals of the alcohol- and acetaldehyde-content of the tissues, and these may be compared with the rates of evolution of 'volatile' carbon occurring at the time. The results are:

	Initially.	After 20 days.	After 30 days.	After 40 days.
Rate of production of 'volatile' carbon (c.c. carbon dioxide per 10 K. hour).	0.2	0.2	1.8	1.5
Alcohol+aldehyde in tissues (as alcohol) (% original fresh weight).	0.016	0.042	—	0.037

It is thus fairly evident that the 10-fold increase in rate of evolution of volatiles that occurs during the climacteric rise in respiratory activity cannot be attributed to an increase in the rate of escape of alcohol or of acetaldehyde.

*Second gathering.* The results obtained with the second gathering, made twenty-one days after the first, are shown in Fig. 2, which deals with them in the same way as that used for the first gathering. In this case the split-current method was not employed, so that we cannot say critically how much higher the average respiratory activity of the bulked fruit used for the chemical determinations may have risen, than did that which is recorded for the isolated individuals.

<sup>1</sup> The mealiness was often accompanied by splitting of the fruit.

As regards the course of respiratory activity, the initial activity after gathering and the pre-climacteric minimum are both lower than in the case of the first gathering, and the climacteric rise begins sooner and proceeds more slowly. The extent of the climacteric rise is again of the order of a 2 to 3-fold increase. The climacteric rise is followed by a further final rise and fall as before. This final rise accompanies breakdown ('sleepiness') but takes longer and reaches a lower maximum than in the case of the first gathering. The fruit of this gathering passed through a juicy state prior to becoming 'sleepy' and finally brown. The juicy stage appeared to be reached between the 30th and 40th day from gathering, and this corresponded with the completion of the climacteric rise and preceded the final rise of respiratory activity associated with breakdown.

The production of carbon, other than that of carbon dioxide, was of the same order of magnitude as that of the fruit of the first gathering and was related to the course of respiratory activity and to ripening and decay in the same way. An approximately 10-fold increase in the rate of production of volatiles occurred during the course of the climacteric rise in respiratory activity, while during the subsequent phase of breakdown the rate of volatile-production diminished. There was again no suggestion that volatiles were predominately alcohol and aldehyde, as the content of the tissues as regards these substances did not change appreciably throughout. The values obtained were: initially 0.017 per cent.; after 13 days 0.029 per cent.; after 41 days 0.03 per cent., and after 52 days 0.03 per cent.

*Third gathering.* The results for the third gathering are shown (Fig. 3). in the same way as those of the first and second gatherings. This gathering was made forty-three days after the first, and twenty-two days after the second. The initial activity is again lower and the climacteric begins sooner and rises more slowly. In this case the split-current method was used from about the middle of the climacteric rise onwards, and the indications are that the activity of the bulked samples rose sooner and to a higher level than that of the isolated fruits. The first removal of individuals from the bulked sample for individual recording was made during the climacteric rise of the bulked samples, and the results with these indicate that their average activity was a good deal higher at that time than that of the individuals isolated since gathering.

There was again no indication that the volatiles which are associated with the climacteric are mainly alcohol and aldehyde.

#### RESPIRATORY ACTIVITY, SUBSTRATE CONCENTRATION AND PROTEIN NITROGEN CONTENT

In the three gatherings the pitch of respiratory activity at the time of gathering falls markedly from August 4 to September 16, but the ratio

respiratory activity/protein nitrogen per unit fresh weight remains approximately constant. Thus:

	Initial respiratory activity at 10° C. c.c. CO <sub>2</sub> per 10 K.H.	Protein content.	Ratio respiratory activity/protein nitrogen $\times 10$ .
First gathering. . . .	83	0.065	128
Second gathering. . . .	51	0.045	113
Third gathering. . . .	41	0.033	124

The ratio, total nitrogen/protein nitrogen, also remains constant.

During storage there is a distinct indication that the protein nitrogen increases, both absolutely and as a percentage of the total nitrogen. The marked rise in the pitch of respiratory activity—the climacteric rise—which takes place during storage is, however, out of all proportion to the rise in nitrogen. The total nitrogen does not appear to change during storage.

As regards other components, we find that both free glucose and acid fall in concentration from the first to the third gathering (Tables V, VI, VII). The fall in respiratory activity is considerably greater than the fall in glucose. In the case of acid, however, the fall in concentration is approximately proportional to the fall in respiratory activity. During storage there is no correspondence between the drifts of respiratory activity and those of any of the components, with the possible exception of sucrose (Fig. 4). In the third gathering there appears to be quite a definite rise in sucrose corresponding to the climacteric rise in respiratory activity, and the results for the other two gatherings, though not so pronounced, are similar. As in the case of protein nitrogen, the rise in sucrose is proportionately far smaller than the rise in respiratory activity.

To sum up, therefore, we can say that in the pre-storage drift on the tree the respiratory activity (per unit fresh weight) is very closely correlated with protein nitrogen content and with acid content. In the storage drifts the climacteric rise is probably accompanied by a rise in the ratio of protein nitrogen to alcohol-soluble nitrogen and in the absolute amount and concentration of cane sugar.

#### LOSS OF CARBON ESTIMATED BY CO<sub>2</sub>-PRODUCTION AS COMPARED WITH LOSS OF CARBON ESTIMATED BY COMBUSTION OF STORED MATERIAL

The estimate of the carbon loss by respiration should be based on CO<sub>2</sub>-production of the bulk of the samples in their storage container. As has been seen, this does not differ significantly during the preclimacteric period from that of individuals withdrawn from the bulk and studied in isolation. Subsequently to the climacteric, however, it does.

For this reason, therefore, in the case of the second gathering we have not a strictly reliable estimate of the CO<sub>2</sub>-production of the storage samples after the 20th day. Values can, however, be reasonably extrapolated up to the 45th day. Three samples were analysed on that day giving an average carbon

loss by combustion of 0.59 gm./100 gm. original fresh weight as compared with 0.563 estimated from  $\text{CO}_2$ -production. The two further samples analysed on the 51st and 59th days give a much higher carbon loss by combustion than

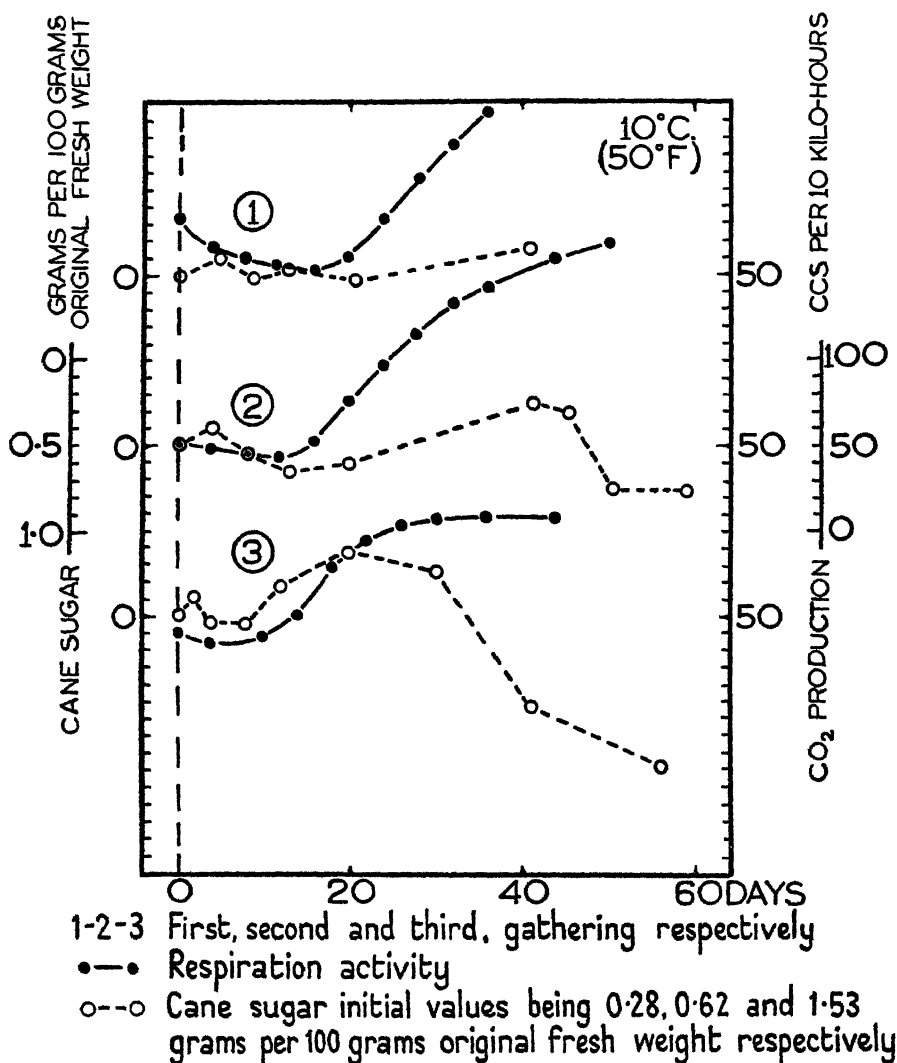


FIG. 4. Storage drifts of cane sugar and respiratory activity.

is to be expected from the measured loss of  $\text{CO}_2$  from isolated individuals, and it is therefore probable that the respiratory activity of these samples rose to high values towards the end of their life and thus behaved in a manner more like those of the first gathering than like those of the third gathering.

In the case of the third gathering there is a close agreement throughout between the loss of carbon estimated by combustion and that estimated by  $\text{CO}_2$ -production.

In the first gathering the agreement is less good. The apparent loss of carbon as estimated by combustion is possibly too small in the early stages.

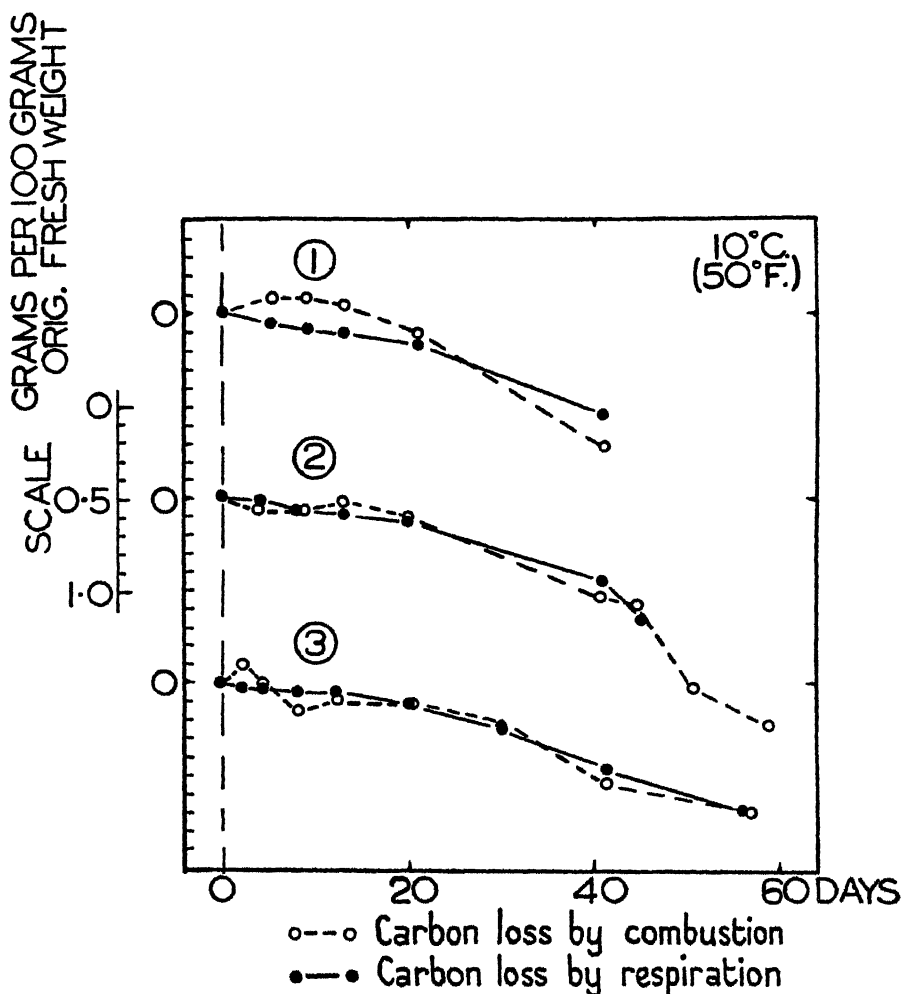


FIG. 5. Comparison of carbon loss as estimated from  $\text{CO}_2$ -production and from combustion.

The four initial samples gave values of 7.24, 7.30, 7.30, 7.24 for total carbon per 100 gm. original fresh weight, while the first three storage samples analysed after 5, 9, and 13 days gave values actually higher than these, i.e. 7.35, 7.34, and 7.30 respectively.

The results for loss of carbon by the two independent methods of estimation are shown graphically for each gathering in Fig. 5.

## DISCUSSION OF CHEMICAL RESULTS

1. *Pre-storage drifts.*

The drift of change occurring in the growth of the fruits on the tree prior to gathering will first be considered. For convenience the relevant data are summarized below in tabular form.

TABLE I

(i) *As Percentage of Fresh Weight*

Date.	Average weight of a fruit.	Glucose.	Fructose.	Sucrose.	Acid.	Starch.	Alcohol-insoluble residue (less starch).	Unestimated alcohol-soluble matter.	Total.	Nitrogen.
Aug. 4 . . .	60.2	1.23	2.66	0.277	0.23	0.48	7.85	4.07	16.80	1.09
Aug. 25 . . .	82.6	1.12	3.72	0.621	0.16	0.48	5.40	3.88	15.38	0.74
Sept. 16 . . .	117.5	0.83	4.47	1.527	0.13	0.34	3.88	3.35	14.36	0.55

(ii) *As g. per average fruit*

Present on Aug. 4 . . .	—	0.74	1.60	0.167	0.14	0.29	4.74	2.46	10.1	0.65
Increase between Aug. 4 and 25 . . .	—	0.18	1.48	0.34	0.00	0.11	-0.27	0.74	2.60	-0.04
Increase between Aug. 25 and Sept. 16 . . .	—	0.05	2.17	1.28	0.01	0.00	+0.09	0.75	4.15	+0.04
Increase between Aug. 4 and Sept. 16 . . .	—	0.23	3.65	1.62	0.01	0.11	-0.18	1.49	6.75	±0.00

It appears from Table I (see also Fig. 6) that during the period of forty-three days from August 4 to September 16 the fruits doubled in size. This doubling in size was evidently due to stretching of the existing cells, while the mass of the cell wall structural elements remained constant. These elements will be included in the fraction described as alcohol-insoluble residue less starch, and consist of cellulose, lignin (mainly in 'stone' cells), hemi-celluloses and pectin. The starch does not show any great change in quantity per fruit, but during the period it is probable that a small net gain may have given way to a small net loss. Glucose and acid per fruit are also relatively constant, showing only a slight increase over the period. There is no significant change in protein nitrogen or total nitrogen per fruit. Thus the considerable increase in dry weight per fruit is almost entirely due to fructose, sucrose, and the unestimated alcohol-soluble material at least half of which, as stated above, is sorbitol.

On gathering, the supply of substance which is being transported via the conductive elements from the tree to the fruits and which forms the raw material for the growth of the fruit is suddenly stopped. It is, therefore, interesting to see if there are any changes in the various components of the fruit which proceed for a short time after gathering and which can be distinguished from subsequent storage-drifts. If this is done (Fig. 6) it appears that the upward drifts that are in progress before gathering in the case of

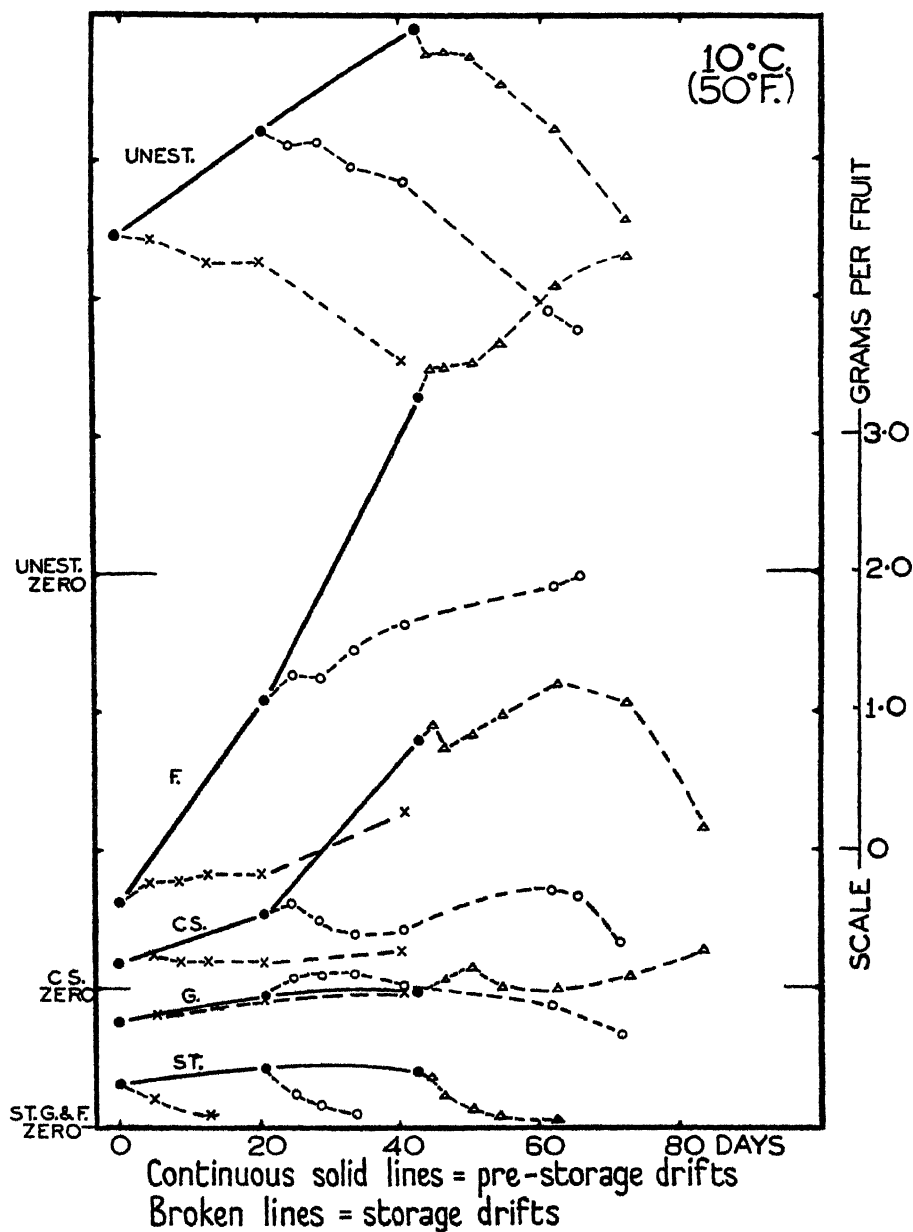


FIG. 6. Pre-storage drifts in various estimated fractions expressed as amounts per fruit plotted together with storage drifts of the successive gatherings similarly expressed.



fructose and sucrose are continued with only slight modification for the first few days. The same is true of glucose, except that in the second and third gatherings the slight increase noticeable as proceeding on the tree is accentuated. In contrast with the above, the upward drift of change in the case of starch and sorbitol is immediately reversed on gathering. These facts suggest that what may be called a 'supply head' of transport sugar is necessary to maintain starch and sorbitol, and that the starch and sorbitol are present in sufficient quantity to maintain for a time after gathering, and even to increase, the amounts of fructose, glucose and sucrose by being converted into those substances.

## 2. Increase in dry weight after gathering.

When allowance is made for the loss of carbon in respiration, the present data show an apparent increase in dry weight after gathering, which is statistically significant. When, however, a further allowance is made for the uptake of water in the hydrolysis of starch this apparent gain is reduced and is no longer statistically significant (Table II). A repetition of the experiment was made and essentially similar results obtained (Table III).

TABLE II

*Gain in Dry Weight after Gathering. Conference Pears. 1936.*

	Gain or loss in dry weight.	Loss of sugar (carbon of resp. $\times 2.5$ ).	Total gain or loss of system.	Increase due to starch hydrolysis.
<i>1st gathering 4th August</i>				
After 5 days	+0.274	0.115	+0.389	Average +0.182 +0.038
" 9 "	-0.189	0.192	+0.003	
" 13 "	-0.035	0.262	+0.227	
" 21 "	-0.226	0.405	+0.179	
" 41 "	-1.230	1.342	+0.112	
<i>2nd gathering 24th August</i>				
After 4 days	+0.064	0.062	+0.126	Average +0.130 +0.035
" 8 "	+0.156	0.122	+0.278	
" 13 "	-0.169	0.195	+0.026	
" 20 "	-0.252	0.342	+0.090	
<i>3rd gathering 16th September</i>				
After 2 days	-0.025	0.025	$\pm 0.000$	Average +0.051 +0.028
" 4 "	-0.069	0.047	-0.022	
" 8 "	-0.099	0.090	-0.009	
" 12 "	+0.006	0.140	+0.146	
" 20 "	-0.166	0.313	+0.147	
" 30 "	-0.649	0.632	-0.017	
" 41 "	-1.004	1.120	+0.116	

TABLE III

*Gain in Dry Weight after Gathering. Conference Pears, 1936. Apparent gain 0.20 of which 0.04 can be accounted for by hydrolysis of starch.*

Number.	Alcohol-insoluble residue.	Alcohol-soluble residue.	Total dry weight.	+ Sugar = Respiratory loss of carbon.
Initial				
I	6.002	10.212	16.214	—
II	6.050	10.200	16.250	—
III	6.024	10.236	16.260	—
IV	6.183	10.150	16.333	—
V	5.938	10.232	16.170	—
VI	5.938	10.345	16.283	—
VII	5.947	10.300	16.247	—
VIII	6.027	10.305	16.332	—
Mean	6.013	10.248	16.261	—
Storage				
5 days				
1	5.725	10.660	16.385	16.465
2	5.767	10.393	16.160	16.240
3	5.763	10.543	16.306	16.386
4	5.857	10.475	16.332	16.412
Storage				
10 days				
5	5.439	10.582	16.021	16.182
6	5.629	11.200	16.829	16.990
7	5.496	10.720	16.216	16.377
8	5.501	10.970	16.471	16.632
Mean	5.647	10.693	16.340	16.460

One possible explanation of an increase in dry weight after gathering is that a volatile substance present in the pear when gathered is converted at 10° C. after gathering into a non-volatile substance. Such a volatile substance would always be lost during the evaporation of the alcohol used for extraction or during the subsequent drying of the residue or of the extract.

It is interesting to note in this connexion the behaviour of the 'non-acid' carbon fraction of the lead acetate precipitate. The values are tabulated in Table IV. They indicate that this fraction is present in greater amount in the stored fruit of all gatherings than in the fruit at the time of picking. There is no definite evidence of a storage drift. While, however, so much may be said, it is nevertheless clear that the increase in dry weight cannot be certainly attributed to this fraction, since the amounts concerned are small and could have been derived from some other fraction.

It is also interesting in the same connexion to note the behaviour of the fraction, residue less starch (see plotting in Fig. 7). The data for the three gatherings show that the main result is the same in each case—an overall loss of residue less starch during storage. There is further, however, a strong suggestion that while in the case of the last picking this loss proceeds immediately on gathering, in the case of the earlier pickings it is progressively delayed. There may even have been in the case of the earliest picking an actual rise to begin with. In explanation of these results it is natural to think in the first place that they may be associated with the progressive delay that occurs

TABLE IV

'Non-acid' Carbon Fraction of the Lead Acetate Precipitate. Conference Pears, 1936.

1st gathering		2nd gathering		3rd gathering	
Initial samples	Storage samples	Initial samples	Storage samples	Initial samples	Storage samples
g./100 g./original	g./100 g./original	g./100 g./original	g./100 g./original	g./100 g./original	g./100 g./original
fresh	fresh	fresh	fresh	fresh	fresh
weight.	weight.	weight.	weight.	weight.	weight.
0.102	0.193	0.102	0.322	0.162	0.304
0.080	0.301	0.196	0.126	0.129	0.150
0.126	0.236	0.168	0.194	0.177	0.143
0.078	0.304	0.178	0.134	0.178	0.072
—	—	0.178	0.258	0.113	0.106
—	—	0.092	0.140	0.054	0.151
—	—	—	0.309	—	0.084
—	—	—	0.108	—	0.038
Average	0.096	0.152	0.199	0.119	0.165

with earlier and more immature gatherings in the onset of the climacteric rise in respiratory activity. It seems doubtful, nevertheless, when we look at the magnitudes involved, whether this explanation can be sufficient. If so, we must conclude that there may have been additions to the fraction residue less starch from one of the other fractions, although the data do not afford any obvious evidence of such, or that the volatile fraction postulated above may have been converted into substances appearing in the insoluble residue.

### 3. Storage Drifts.

(a) *Total fructose and sorbitol.* Turning now to a consideration of the drifts of change during storage after gathering, attention may be first directed to the most obvious and quantitatively largest feature of the results. This feature is the large decrease in the unestimated fraction and the almost equivalent increase in total fructose. The data in this respect are shown graphically in Fig. 8. It may be remarked here that owing to the relatively small changes in sucrose during storage in the present case the values for gains in total fructose closely represent those for free fructose.

It seemed advisable, however, to test whether there might not in fact be some other substance present which would appear in our estimations as fructose, that is to say, a substance that would reduce copper, would not be oxidized by iodine, and was fermentable. Accordingly osazone identification was carried out. The cleared alcoholic extract of a sample of the second gathering after forty-one days' storage gave a yield of osazone crystals, equivalent to about 81 per cent. of the hexose expected from the analytical data. Nothing

but typical crystals could be seen under the microscope. The melting-point was  $203^{\circ}\text{C}$ . and was not changed after recrystallization. An artificial mixture of glucose and fructose in the same relative proportions as those expected from the analytical data, was then treated in the same way. The yield was

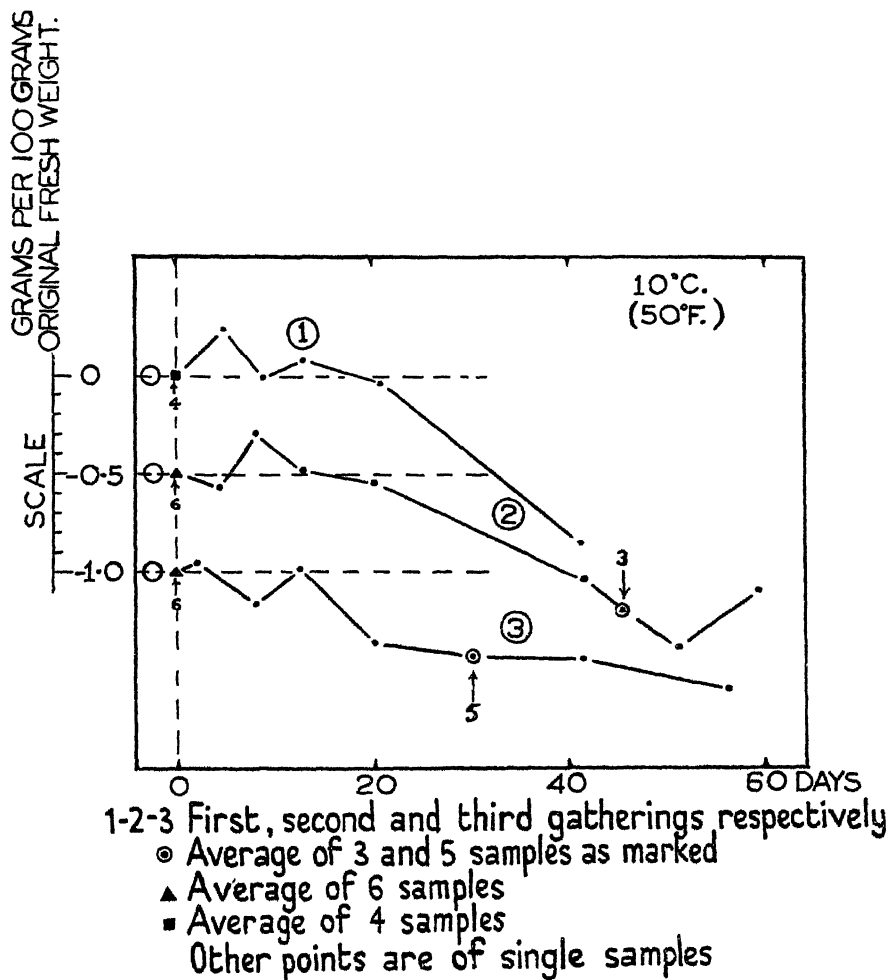


FIG. 7. Storage drifts of alcohol insoluble-residue.

83 per cent. of hexose and the melting-point  $204^{\circ}\text{C}$ . We conclude, therefore, that some component of the unestimated fraction is quantitatively converted to fructose in the pear during storage at  $10^{\circ}\text{C}$ . The few estimations of sorbitol that were made indicate that the main loss of the unestimated fraction is a loss of sorbitol.

Closer inspection of these data (Table VIII) indicate that while the gains on

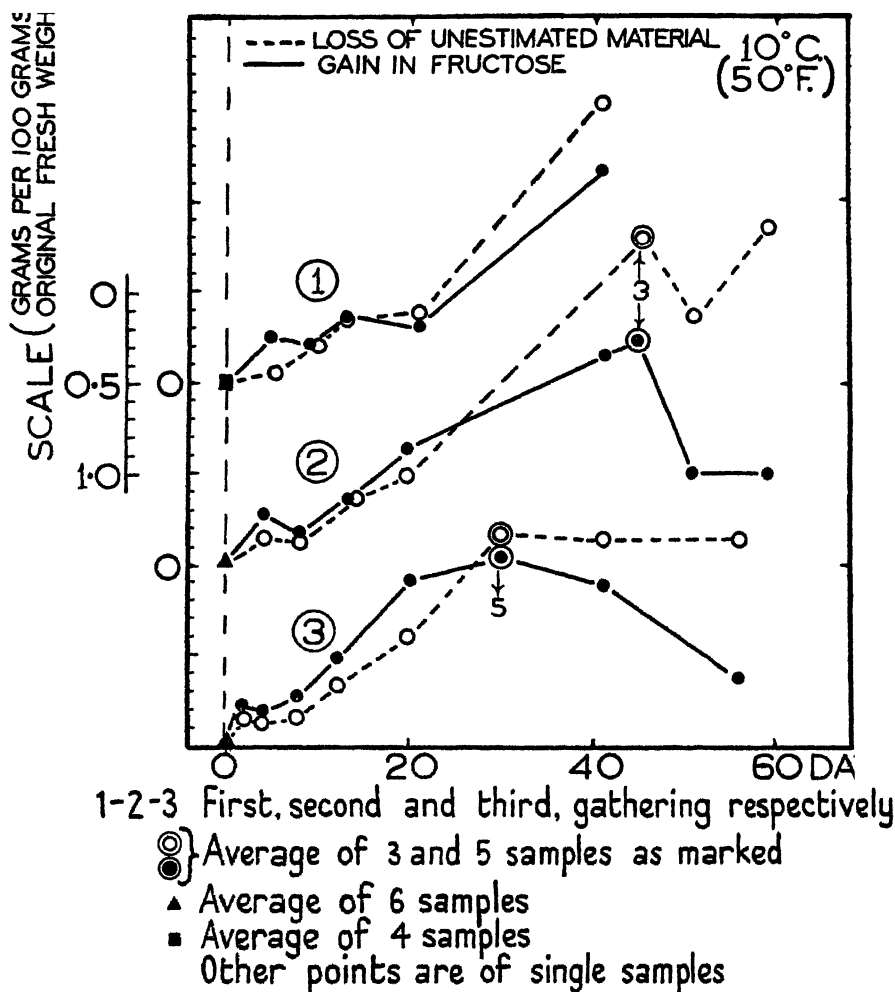


FIG. 8. Change in amount of (i) unestimated alcohol-soluble fraction containing sorbitol, and of (ii) total fructose units.

the one hand and the losses on the other are approximately equivalent, the three gatherings behave systematically in the following respects:

1. There is at first a small excess gain of 'total fructose' which is more marked in the second and third gatherings.
2. After 15 to 20 days' storage, loss of unestimated material exceeds gain of fructose.
3. In the later stages, i.e. after 30 days' storage, 'total fructose' ceases to increase and begins to decrease.





TABLE VIII

Days	1st gathering		Days	2nd gathering		Days	3rd gathering	
	Total fructose gain.	Unestimated fraction loss		Total fructose gain.	Unestimated fraction loss		Total fructose gain	Unestimated fraction loss
5	0.250	0.059	4	0.262	0.124	2	0.225	0.146
9	0.206	0.205	8	0.167	0.117	4	0.175	0.122
13	0.358	0.343	13	0.369	0.336	8	0.262	0.156
21	0.333	0.346	20	0.621	0.461	12	0.457	0.316
41	1.163	1.536	41	1.148	1.576	20	0.903	0.614
			45	1.209 (3)	1.793 (3)	30	1.034 (5)	1.163 (5)
			51	0.531	1.374	41	0.887	1.119
			59	0.483	1.848	56	0.366	1.132
% present at outset	2.794 (4)	4.069 (4)		4.030 (6)	3.878 (6)		5.229 (6)	3.327 (6)

The figures in brackets indicate the number of replicate samples.

These facts link up with certain conclusions from apple data obtained in this laboratory. These are: (1) that fructose units are formed from starch during its hydrolysis in the plant; (2) that provided the rate of respiration of sugar does not exceed about half the rate of the production of sugar from starch, the fructose units formed accumulate both as free fructose and in combination as sucrose; and (3) that free fructose does not enter the system as an immediate substrate of respiration until after the climacteric, and up to that time may be regarded as an end product. With the present data the progress of sugar loss calculated from the carbon of respiration may be compared with the progress of starch hydrolysis in Fig. 9, from which it appears that the initial rate of production of sugar from starch is about double the rate of sugar loss in respiration in the first gathering, while in the third gathering it approximates to three times the rate of respiratory consumption, the second gathering being intermediate in this respect. We should therefore expect some formation of 'fructose', more especially in the second and third gatherings, during the period of starch hydrolysis in addition to that formed from the unestimated fraction.

(b) *Total glucose, residue less starch and respiratory drain.* In Figs. 10 and 11 the data are examined from the angle of the drifts in total glucose and in residue less starch compared with the drift of respiratory loss of carbon (as sugar). The third gathering will be considered first.

There is, as might be expected, for about five days during the period of starch hydrolysis a greater loss of total glucose than can be accounted for by respiratory drain. This is attributed to the formation of fructose units from glucose units during the hydrolysis of starch. Then for a period of about twenty days total glucose does not change. On the other hand, from the outset to the end of this period respiratory drain corresponds very closely with loss of residue. It is suggested, therefore, that residue less starch is either



being respired directly or is yielding a sugar estimated as glucose (i.e. a fermentable reducing sugar, oxidized by iodine). Subsequently the respiratory

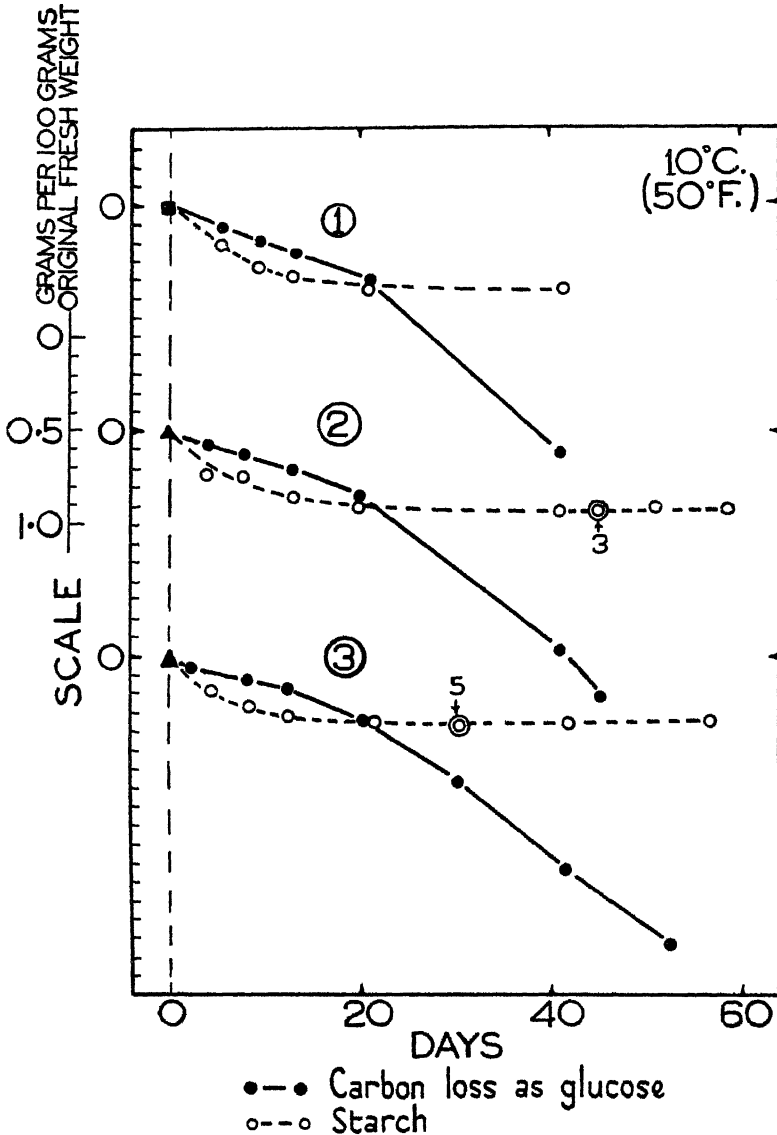


FIG. 9. Respiratory drain compared with hydrolysis of starch.

drain clearly exceeds loss of residue and at the same time there is a loss of total glucose, while total fructose gain falls short of the loss of unestimated material as indicated above. The picture thus formed is one in which the

substance drawn upon throughout in respiration is glucose, fructose becoming an additional substrate during the period following the climacteric. In the present case an essential feature in the picture is that the non-starch fraction of the alcohol-insoluble residue is continually producing glucose, or a sugar estimated as glucose, and it is accidental that for a time the rate of production of these total glucose units equals the rate of respiratory drain upon glucose.

In the first two gatherings the results for the drifts of total glucose, residue less starch and of respiratory loss of carbon (as sugar) set out in Figs. 10 and 11 are complicated by an additional factor which seems to be connected with the phenomenon referred to above, namely, that residue less starch does not fall from the outset but exhibits an initial phase in which there is either no loss or an actual gain. Comparing only total glucose loss and respiratory drain it appears that they are strikingly equivalent for about ten days in the case of the first gathering and for about twenty days in the case of the second gathering. This appears to afford good evidence that the respiratory drain during this time is a drain upon glucose alone. Such a conclusion, however, does not harmonize with the fact that there is an excess production of fructose units over loss of unestimated material, nor does it help in any way to explain the anomalous behaviour of the residue less starch fraction in the case of these two immature gatherings.

In the later stages of the storage drifts the total glucose fraction, in the case of the first gathering, ceases to fall, remains level for a time and then actually rises. During this period it is clear that the respiratory drain must be largely met, directly or indirectly, from the residue fraction. The loss of residue is practically equivalent to the loss of carbon (as sugar) in respiration. The rise in total glucose, on the other hand, is approximately equivalent to the amount by which the total fructose fraction falls short of the unestimated fraction. It does not seem possible to deduce from the data whether fructose in the total fructose fraction is respired directly or converted into glucose. If, however, it is respired directly, the rise in total glucose must be due to the formation from the residue fraction of glucose units or, to be more precise, of units of a reducing fermentable sugar oxidizable by iodine.

In the case of the second gathering the loss of residue in the later stages of the storage drift is insufficient to account for the respiratory drain. Here we find, as in the case of the third gathering, a drain in addition on total glucose and on fructose.

If the residue less starch is in fact producing units estimated as glucose during the later stages it may well be doing so all along, as we have thought is probably the case with the third gathering. If so, the net loss in glucose units in the early stages is due to consumption in respiration and to conversion to fructose during starch hydrolysis in excess of production from residue. The parallelism between net glucose loss and respiratory drain may therefore be fortuitous. The course of the drift of the residue values which shows either no fall or a rising tendency during the early stages, must then be attributed to

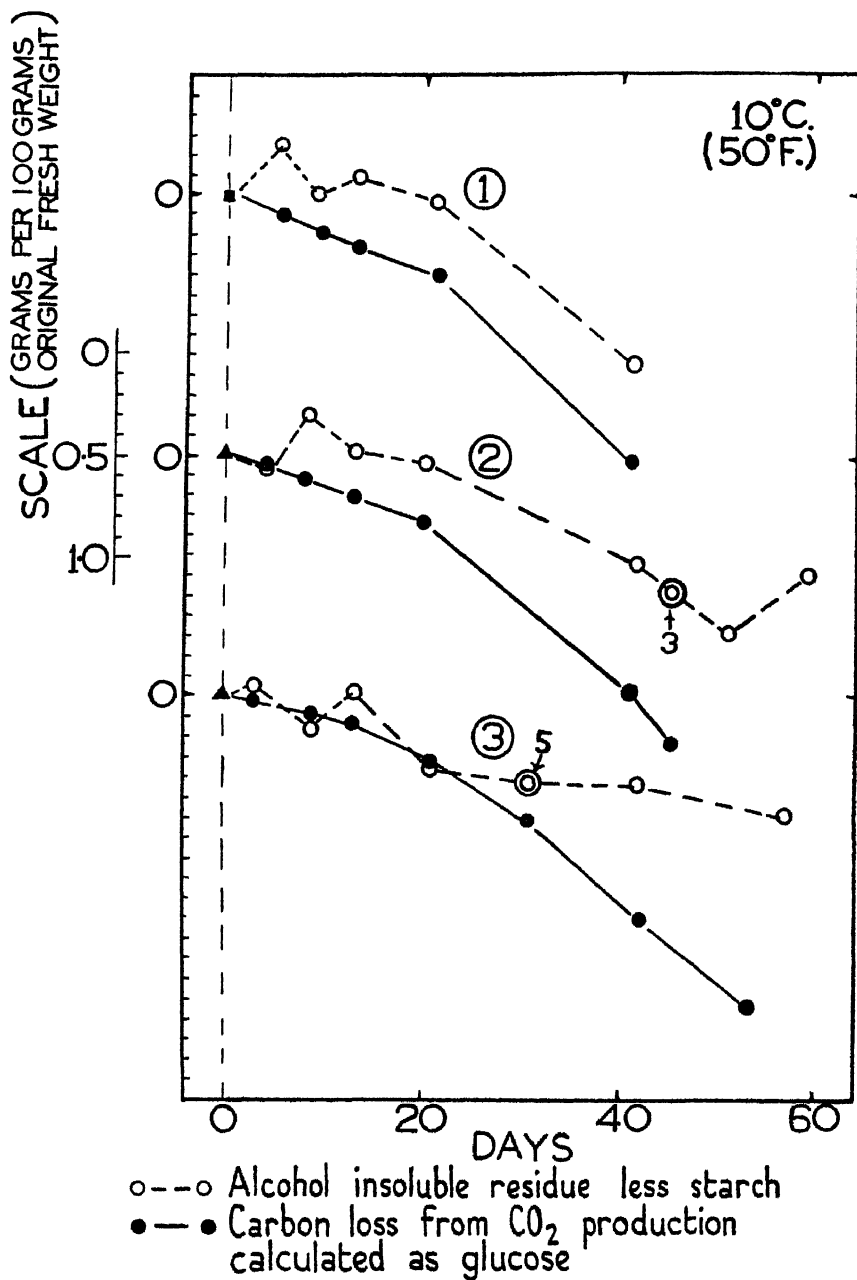


FIG. 10. Respiratory drain and residue loss compared.

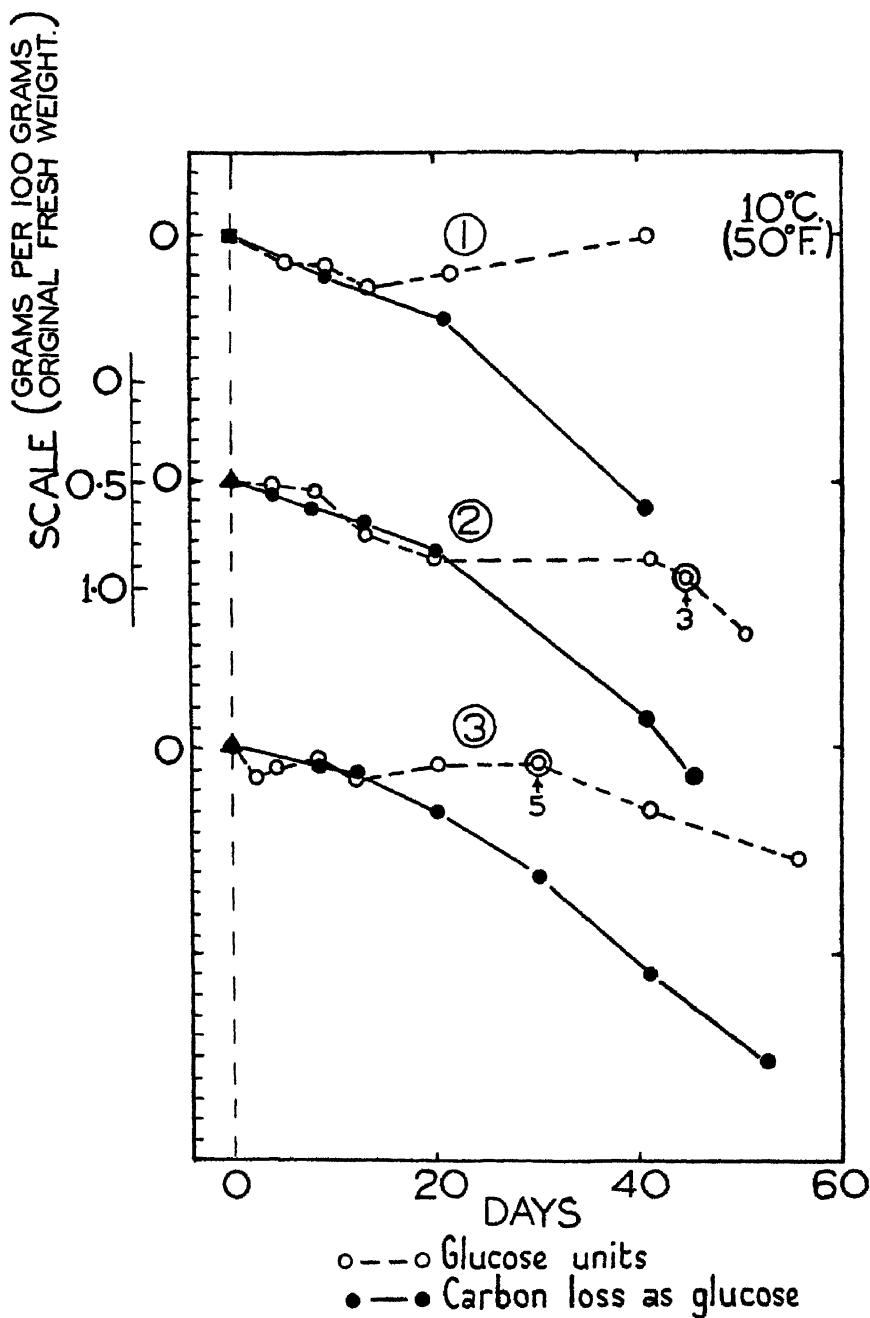


FIG. 11. Respiratory drain and loss of glucose units compared.

the conversion into alcohol-insoluble substances of some volatile substance present in the tissues.

To sum up, therefore: our conclusions from a close scrutiny of the storage drifts of total fructose units, total glucose units, sorbitol and alcohol-insoluble residue less starch are that the residue produces alcohol-soluble units estimated as glucose, and that the respiratory drain is in the pre-climacteric stage solely upon glucose, and subsequently upon both glucose and fructose.

(c) *Acid*. As compared with the apples which we have studied (Bramley's Seedling and Worcester Pearmain) the acid content of the Conference pear is low. In apples there is generally a progressive loss of acid during storage, the form of the curve being a logarithmic decline. In certain cases, however, there are departures from this form; thus for a short time at the beginning of storage during the period of starch hydrolysis there may be no loss of acid, and when physiological breakdown occurs in cold storage, the rate of loss of acid increases.

The drift of acid in pears during storage is set out for the three gatherings in Fig. 12. There seems to be no significant change in acid until physiological breakdown commences. Subsequently there is a definite decrease in the content of acid.

#### SUMMARY

An account is given of the chemical changes occurring during storage in air at 10° C. of Conference pears, gathered at three stages of maturity. The respiratory activity of the fruit was determined from the time of gathering onwards and is discussed in relation to the observed chemical changes.

The curves for respiratory activity (expressed as rate of CO<sub>2</sub>-production per unit fresh weight) are essentially similar to those for apples. They show, after gathering, an initial falling rate. At maturity the rate begins to increase (the climacteric rise), the extent of the rise eventually being of the order of a 2 to 3-fold increase. The climacteric rise is followed by a final rise and fall, the final rise being accompanied by the breakdown of the flesh of the fruit. The final fall in rate, which, in the absence of fungal infection, would probably reach zero activity, is associated with the death of the tissue.

The rate of production of carbon from volatile substances (other than CO<sub>2</sub>) showed an approximately 10-fold increase during the course of the climacteric rise in respiratory activity. During the subsequent phase of breakdown of tissues a marked falling off in rate was observed. There was no evidence that the volatile substances were predominantly alcohol or aldehyde.

In the pre-storage drift on the tree the respiratory activity of the fruit is very closely correlated with the content of protein nitrogen and of acid. In the storage drifts the climacteric rise is probably accompanied by a rise in (i) the ratio of protein nitrogen to alcohol-soluble nitrogen and in (ii) the absolute amount of cane sugar.

During storage there is a marked decrease in the unestimated fraction and

an almost equivalent increase in fructose units. The unestimated fraction is approximately 4 per cent. of the fresh weight. About half this fraction consists of sorbitol, and the decrease during storage can be accounted for by the loss of sorbitol.

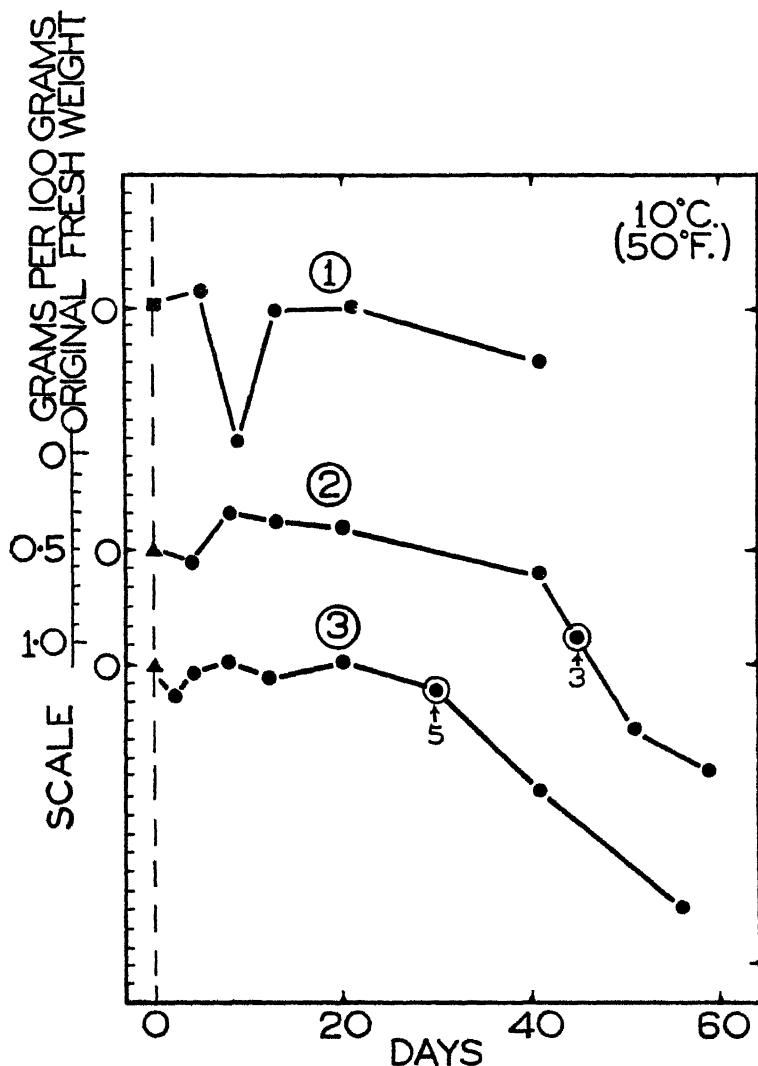


FIG. 12. Storage drifts of acid calculated as malic acid.

In discussing the relation of the respiratory activity of the fruit to the observed chemical changes, attention has been concentrated on the carbohydrate changes since the acid content is very small. These changes have been interpreted as being the result of (i) a respiratory drain on the glucose units

throughout; (ii) a supplementary drain on fructose units during the post-climacteric phase; (iii) the formation of glucose units or of units estimated as glucose, from the alcohol-insoluble residue, and (iv) the formation of fructose units from glucose units during the hydrolysis of starch.

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# The Response of Seeds and Seedlings to Treatment with Indolylacetic Acid

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With Plate I

## INTRODUCTION

A CONSIDERABLE amount of information has recently accumulated as to the effect on the growth of plants of applying indolylacetic acid and other synthetic plant hormones to seeds and to whole plants. Cholodony (1936), Grace (1937), Amlong and Naundorf (1937, 1938, 1939 and 1939*a*), Thimann (1938), and Thimann and Lane (1938) have all reported increased growth of various species of plants after treating the seed with appropriate doses of such hormones. Davies, Atkins, and Hudson (1937), and Templeman (1939), however, failed to obtain such stimulation using similar treatments. Hitchcock and Zimmerman (1935) found shoot growth to be retarded by applying to the soil of pot-grown plants relatively high concentrations of hormone solutions, and Pearse (1936, 1937 and 1937*a*) and Templeman (1939) failed in several species to obtain increases in dry weight by supplying hormones in the culture medium or to the plants by spraying. Grace (1937*a* and *b*), Amlong and Naundorf (1938), Greenfield (1937), and Loehwing and Bauguess (1936), however, have reported growth increases of greater or lesser extent by treatment with optimal amounts of hormones.

It is very evident that there is great variability in the effect on the growth of plants of a treatment of either the seed or the whole plant with synthetic hormones. Little further progress can be made until the underlying causes of this variation are revealed. The present paper describes firstly, attempts to obtain the stimulating effect of seed and soil treatment with indolylacetic acid, and, secondly, attempts to throw light on the possible factors governing the response to these plant hormones by the use of other treatments simultaneously with the hormone treatment.

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## MATERIAL AND METHOD

The indolylacetic acid solution used in these experiments was prepared by dissolving the substance in a small amount of 95 per cent. alcohol (Hitchcock and Zimmerman, 1936), made up to the required volume with tap-water.

Oats (*Avena sativa*) and Broad Bean (*Vicia Faba*) seedlings were used as test plants. Seeds were germinated in moist Petri dishes, and later planted in 4-inch pots containing garden soil or silver sand, or transferred to blackened glass bottles containing a nutrient solution. The solution used had the following composition:  $\text{KNO}_3$  1.0 gm.;  $\text{MgSO}_4$  0.5 gm.;  $\text{CaSO}_4$  0.5 gm.;  $\text{NaCl}$  0.5 gm.,  $\text{KH}_2\text{PO}_4$  0.5 gm.;  $\text{FeCl}_3$  trace; water 1,000 c.c. Throughout the experiment the plants were kept in a greenhouse at a temperature of about 20° C., and those in sand or soil watered every day.

*Experiment 1. The effect of seed treatment.*

The seeds were soaked for twenty-four hours in tap-water containing various concentrations of heteroauxin; 40 to 50 young oat seedlings were then planted in each pot of silver sand watered with nutrient solution. They were harvested after one and two weeks. The data in Table I shows that by

TABLE I  
*Avena sativa* Seedlings in Sand Culture.

Concentration of indolylacetic acid.		Shoot.		Root.		Total		Shoot — Root ratio.	
		Dry wt. (mg. per plant.)		Dry wt. (mg. per plant.)		Dry wt. (mg. per plant.)			
		A	B	A	B	A	B		
Control,	. .	10.7	22.6	7.5	13.9	18.2	36.5	1.4	1.6
2 p.p.m.	. .	11.4	19.6	8.2	12.0	19.6	31.6	1.4	1.6
20 "	. .	8.9	17.8	8.5	12.2	17.4	30.0	1.0	1.5
200 "	. .	9.1	18.2	8.8	13.4	17.9	31.6	1.0	1.4

\* A = after one week, B = after two weeks.

the end of the first week the dry weight of the shoots of treated plants had slightly decreased and that of the roots had increased, changing the shoot to root ratio from 1.4 (in the control and 2 p.p.m. treatment) to 1.0 (in the 20 p.p.m. and 200 p.p.m. treatment). When harvested, however, after two weeks the difference between the shoot/root ratio of the treated and control plants had diminished. Another experiment with broad beans and oats was carried out in water culture and the plants harvested after three weeks. It is evident from Tables II and III that the seed treatment had no effect on the growth of oats, and the broad bean showed a slight growth acceleration at low concentrations and a definite retardation at higher concentrations.

*Experiment 2. The effect on their response to indolylacetic acid of decapitating the young shoots of young oat seedlings.*

Ten to fifteen oat seedlings were planted in each of 4-inch pots containing silver sand and watered with a nutrient solution. After four weeks when the

TABLE II

*Avena sativa. Seedlings in Water Culture.*

Concentration of indolylacetic acid.	Shoot Dry wt. (mg. per plant.)	Root Dry wt. (mg. per plant.)	Total Dry wt. (mg. per plant.)	Shoot— Root ratio
Control . . . .	42.5	18.4	60.9	2.3
2 p.p.m. . . . .	39.2	16.8	56.0	2.3
20 „ . . . . .	40.5	18.6	59.1	2.2
200 „ . . . . .	43.2	18.2	61.2	2.4

TABLE III

*Vicia faba. Seedlings in Water Culture*

Concentration of indolylacetic acid.	Shoot Dry wt. (mg. per plant.)	Root Dry wt. (mg. per plant.)	Total Dry wt. (mg. per plant.)	Shoot— Root ratio
Control . . . . .	429.2	167.3	596.5	2.6
2 p.p.m. . . . .	485.4	182.5	667.9	2.7
20 „ . . . . .	462.7	162.6	625.3	2.8
200 „ . . . . .	286.6	125.4	412.0	2.3

axillary buds of the first leaf were formed, 25 c.c. of heteroauxin solution in tap-water containing 0.1, 1.0, 10, 100 part per million (p.p.m.) were added to each pot. Tap-water alone was given to the control pot. After twenty-four hours the shoots were decapitated in two ways: (1) 1 cm. above the first node, (2) just below the second node. The remaining shoots in the first group were about 1.5 to 2 cm. and in the second 5 to 6 cm. long.

Immediately after cutting the inner leaves protruded from the cut surface due to the release of tension; this was noticed in all the treated and control plants. On the second and third day respectively, development of the buds of the plants treated with 10 p.p.m. and 1 p.p.m. was noticed, while the control, the 0.1 p.p.m. and the 100 p.p.m. treatments showed similar development on the fourth day. In group 2 all the young buds came out at the top of the decapitated shoot on the fifth day, i.e. the buds had grown out to a length of 5 to 6 cm. long.

Plate I, Figs. 1 and 2, show the development of the buds ten days after treatment. At the end of sixteen days the buds were harvested and their lengths and dry weights were determined; these are given in Table IV.

From the figures from Table IV it is evident that the addition of the dilute heteroauxin solution to the sand increased both dry weight and length of buds in the group where the decapitation was just above the bud, but in group 2 where the decapitation was 5–6 cm. above the buds there was no appreciable effect of treatment.

In another experiment 25 c.c. of solutions of indolylacetic and of the same concentration were applied through the cut end of the shoots immediately after decapitation. The response of these seedlings was similar to those in which the hormone solutions were applied directly to the soil before decapitation.

*Experiment 3. The effect of nitrogen deficiency.*

Using the same technique as in the previous experiments, oat seedlings were raised in sand and in garden soil. The nutrient solution used in sand culture was similar to that in experiments 1 and 2, except that the nitrogen was omitted. Plants were decapitated when 15 days old, and at this age the

TABLE IV

*Avena sativa. Response of Decapitated Seedlings to Treatment with Heteroauxin*

Distance of decapitation above soil level.	Concentration of indolylacetic acid.	Length	Dry weight per bud.
		cm.	mg.
Above 2 cm.	Control	6.5	3.2
	0.1 p.p.m.	8.9	4.9
	1     "	12.6	8.2
	10    "	13.2	9.3
	100   "	7.5	4.3
Above 6 cm.	Control	13.8	8.5
	0.1 p.p.m.	14.2	9.6
	1     "	15.0	8.8
	10    "	14.6	8.2
	100   "	15.6	8.5

seedlings in sand were showing definite symptoms of nitrogen deficiency; 25 c.c. of heteroauxin solution of various concentrations were added twenty-four hours before decapitation. The shoots of the seedlings grown in soil were cut in two ways: (1) close to the soil surface and, (2) about 2 cm. above the soil. The seedlings grown in sand without nitrogen supply were all cut 2 cm. above the sand.

From Pl. I, Figs. 3 and 4, and Table V it is clear that the plants grown in soil show distinct response to heteroauxin treatment both with the shoots decapitated just above and 2 cm. above the soil. The stems of plants grown in sand in the absence of nitrogen were weak, bore yellowish-green leaves and did not respond to heteroauxin treatment. In the case of plants grown in soil treated with 100 p.p.m. indolylacetic acid, an acceleration of bud growth was noted on plants which were cut back 2 cm. above soil-level, but a retarding or lethal effect when cut at soil-level.

## DISCUSSION

Under the conditions of these experiments the treatment of seeds of *Avena sativa* with dilute solutions of heteroauxin previous to planting had little effect on the subsequent growth of the seedlings, and higher concentrations caused only inhibition. This result is in agreement with that of Templeman (1939); but at variance with that of Cholodony (1936) Grace (1938) and Amlong and Naundorf (1938). The cause of this variance is still obscure, but the most probable explanation would seem to lie in some difference in the external conditions during culture.

It seemed possible that the failure to respond to applied auxin might be due to the fact that under the culture conditions employed the supply of auxin was never a controlling factor in shoot growth. With this in mind a series of experiments was undertaken in which an attempt was made to remove the main source of natural auxin supply by decapitating the young

TABLE V

*Avena sativa*. Response of Decapitated Seedlings to Heteroauxin.

Method of culture.	Distance of decapitation above culture medium.	Concentration of indolylacetic acid.	Length.	Dry weight of plant.
			cm.	mg.
Soil	0.5 cm.	Control	7.5	4.7
		1 p.p.m.	9.2	6.7
		10 "	13.0	9.6
		100 "	3.3	3.0
Soil	2 cm.	Control	14.6	11.8
		1 p.p.m.	16.5	12.5
		10 "	22.8	20.7
		100 "	18.9	17.6
Sand No nitrogen	2 cm.	Control	15.2	9.2
		1 p.p.m.	14.0	9.8
		10 "	15.6	8.9
		100 "	15.2	9.5

growing shoots and leaves, and determining the effect of applied heteroauxin on the subsequent regeneration. It was then found that the addition of dilute heteroauxin solution to the soil or sand in which the decapitated seedlings were growing resulted, at the appropriate concentration, in an accelerated development of the cut terminal shoots and also of the lateral buds. The magnitude of this acceleration depended on the concentration, and showed a typical optimum curve. Two possible explanations of this result would seem to present themselves: (1) the addition of heteroauxin restores the concentration of auxin in the buds required for optimum growth; (2) the removal in the decapitated stems of inhibiting substances which are retarding regeneration.

It is clear from the data, however, that the response to applied heteroauxin is influenced by the level of decapitation. i.e. the longer the portion of shoot remaining the less the response to applied heteroauxin. Thus when 5 to 6 cm. of shoot remained above the soil there was no difference in the bud development in the treated and the untreated seedlings; moreover, development was then in all cases, as rapid as in any of the seedlings cut at soil-level. This result militates against the theory of a removal of inhibiting substances in the stem, for it would be expected that leaving a longer piece of stem would result in greater inhibition.

The more likely explanation would therefore seem to be that the longer stem piece is able to start producing its own auxin more quickly so that apart

from an external supply the appropriate concentrations for bud growth is soon restored. In favour of this view it is seen in Table I that with the longer stem piece there is a slight response to a very low concentration 0.1 p.p.m. On this theory, therefore, provided the appropriate low concentration of auxin is maintained in the dormant bud it will develop. It has been suggested by Went (1939) that attempts to explain bud inhibition by a direct effect of auxin fail because the longer the piece of stem above the bud the greater the inhibition occurring when heteroauxin is applied to a cut stem above the bud. This objection fails, however, if one assumes that the piece of stem itself produces auxin and the longer the piece the more auxin it produces. Thus the application of auxin will more quickly result in a supra-optimal amount reaching the bud with consequent inhibition. Thus it would seem clear that an increased growth response of a bud to auxin can appear only if that bud is deficient in auxin.

The plants grown in the absence of nitrogen showed no response to auxin treatment, although it has been shown by Avery *et al.* (1936, 1937) that nitrogen-deficient plants produce very little auxin. This failure to respond would therefore seem to be due to the fact that the nutritional factor and not the auxin is in this case limiting development.

#### SUMMARY

The treatment of seeds of Oats (*Avena sativa*) and Broad Bean (*Vicia Faba*) with dilute solutions of indolylacetic acid for twenty-four hours before planting did not result in increased growth (dry weight) of the seedlings. Higher concentrations retarded the growth.

The rate of regeneration of lateral buds and shoots of decapitated oat seedlings growing in sand or soil cultures was increased by adding dilute indolylacetic acid to the cultures. Both the length and the dry weight of the regenerating buds were increased.

This response was influenced by the length of shoot remaining on the seedlings.

Nitrogen-deficient plants showed no such response to heteroauxin treatment.

The bearing of these results on bud inhibition is discussed.

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## EXPLANATION OF PLATE I

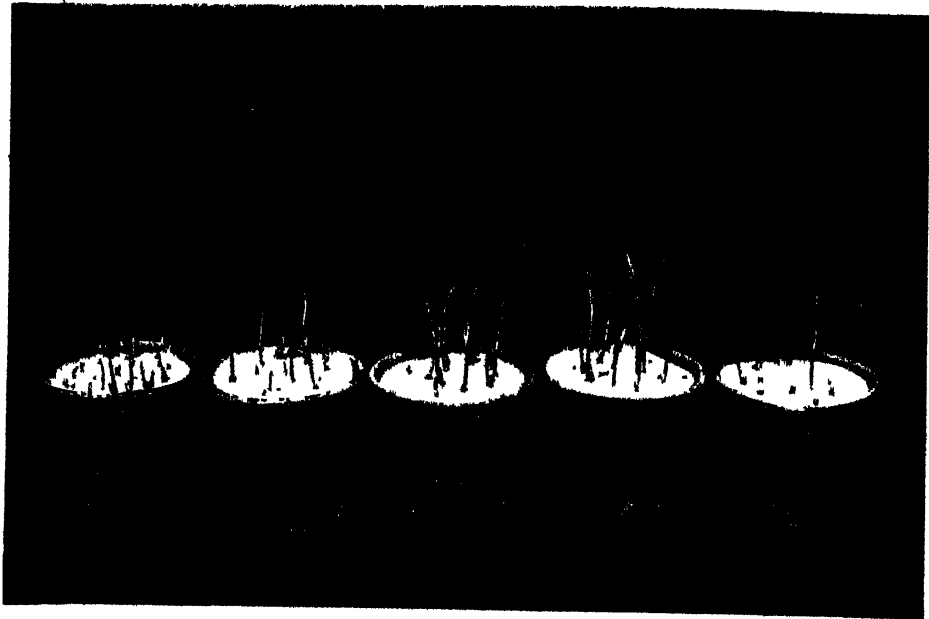
Illustrating Dr. Y. Hwang and Dr. H. L. Pearse's article on 'The Response of Seeds and Seedlings to Treatment with Indolylacetic Acid'.

Fig. 1. *Avena sativa*. Seedlings 4 weeks old decapitated 2 cm. above sand. Left to right: control (tap-water), 0.1, 1, 10, and 100 p.p.m. indolylacetic acid. 25 c.c. applied to sand twenty-four hours before decapitation.

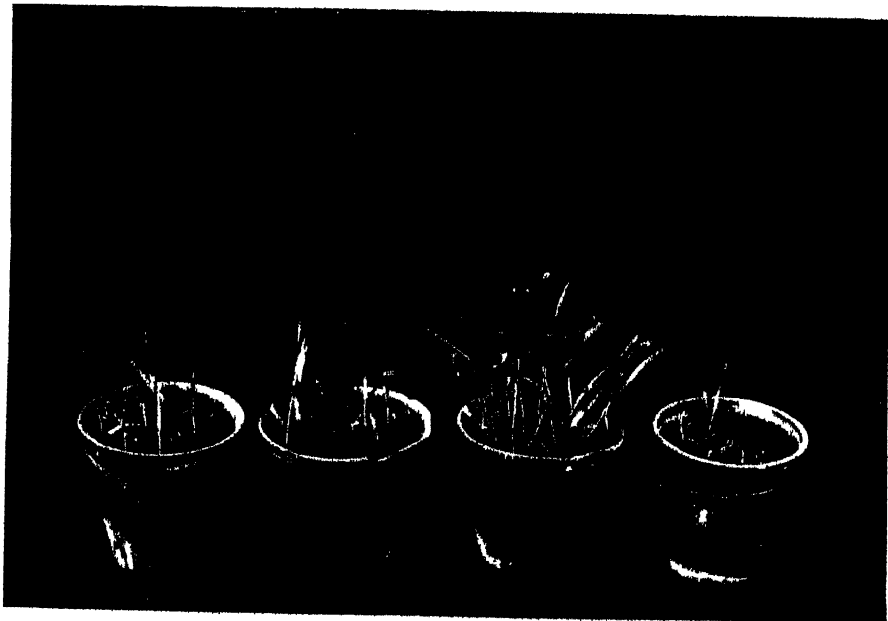
Fig. 2. *Avena sativa*. Seedlings 4 weeks old decapitated 6 cm. above sand. Left to right: treatments as Fig. 1.

Fig. 3. *Avena sativa*. Seedlings 2 weeks old decapitated 0.5 cm. above the soil. Left to right: control (tap-water), 1, 10, and 100, p.p.m. applied to the soil twenty-four hours before decapitation.

Fig. 4. *Avena sativa*. Seedlings 2 weeks old. Above: grown in sand without nitrogen and decapitated 2 cm. above the sand. Left to right: control (tap-water), 1, 10, and 100 p.p.m. indolylacetic acid added to the sand twenty-four hours before decapitation. Below: soil cultures, treatments as above.



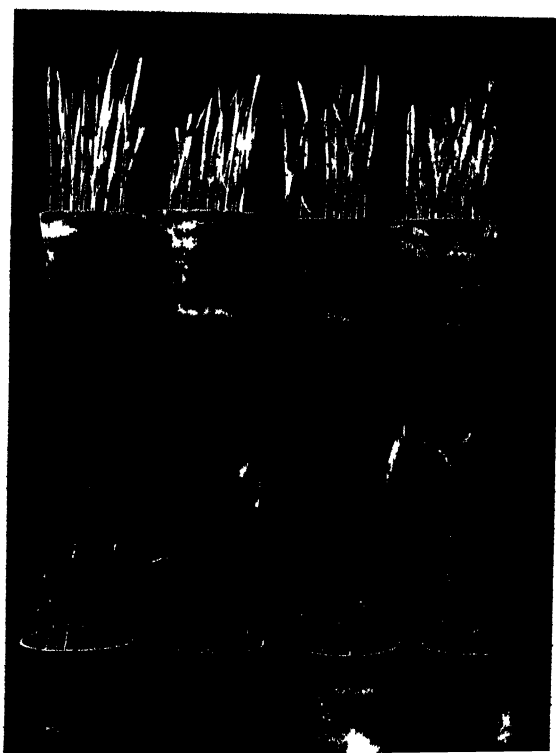
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# Studies in Tropical Fruits

## VIII. Carbohydrate Metabolism of the Banana Fruit during Development

BY

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With thirteen Figures in the Text

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### I. INTRODUCTION

THE stage of maturity at which the Gros Michel banana is harvested for different markets—'¾-full' and 'heavy ¾-full' for the English and North American trades respectively—is assessed in the first instance by direct observation of the size and appearance of the bunch and individual fruits or

fingers, but local knowledge of the rate of development of the bunch and of its chronological age is also essential (Wardlaw, Leonard, and Barnell, 1939). Thus although the size of bunch selected for a particular grade varies from country to country in relation to prevailing local conditions, the general type of fruit harvested for export from any one area tends to be fairly uniform and values may accordingly be assigned the weights of average '¾-full' and 'heavy ¾-full' fruit. In an earlier paper (Wardlaw, Leonard, and Barnell, 1939) data were submitted showing that when the bunch is allowed to remain on the plant, growth continues until the bunch is considerably larger and heavier than the normal export grades.

The purpose of the present paper is to present biochemical data for the Gros Michel banana during development on the plant, to define the biochemical status of standard export and heavier grades for relation with their storage behaviour, and also to ascertain the maximum size attained by fruits before ripening processes are initiated while still on the plant. As a sequel to the investigations described here, studies of the biochemical processes involved in the ripening of bunches cut at various grades and given various storage treatments have been made, and will be submitted in due course. A somewhat similar procedure has given important and useful results in the study of apples, Haynes and Archbold (1929), Archbold (1930), Archbold and Widdowson (1931), Archbold (1932), in which the striking differences in storage behaviour between prematurely and normally gathered fruits of certain varieties led to the investigation of the chemistry of fruit development from petal-fall until after normal gathering time with a view to defining 'maturity' in chemical terms.

In the past, studies in the biochemistry of the banana have been almost entirely confined to the ripening of fruits which had already undergone a period of transport, refrigerated or otherwise, e.g. Poland, Manin, Brenner, and Harris (1938), Bourdoul (1931), Stratton and von Loesecke (1930), Gane (1936), Gore (1914). Belval (1930), however, studied the formation of starch in the fruit, estimating the carbohydrates of other parts of the plant simultaneously, and reached the conclusion that the starch in the fruit was formed principally at the expense of the reducing sugars delivered by the peduncle, the sugar in the fruit being mainly sucrose.

## II. METHODS AND PROCEDURE

Throughout the present study, analyses have been made of the entire skin and entire pulp of single fingers.<sup>1</sup> Preliminary results obtained from analyses of individual fingers selected as having attained corresponding levels of maturity showed sufficient uniformity to justify the use of single finger

<sup>1</sup> This work was carried out before the reconditioning and extension of the Trinidad Low Temperature Research Station was completed and, in the absence of refrigeration, the sampling of bulked quantities of fruit proved impracticable because of the high temperature of the laboratory and the time required to slice or pound the material into sufficiently small pieces to ensure thorough mixing.

sampling in this investigation. In Table I are given the means of observations made on the skin and pulp of fingers taken from hands ranging from the 1st to the 9th of three bunches. The first set of samples (A and C) was taken on the day of arrival when the fingers were green and hard and the second set (B and D) after an interval of eight days when they were yellow and soft. The values given in the table for the standard deviations of individual observations

TABLE I  
*Analysis of Single Fingers. (Per cent. of fresh weight).*

	Total dry matter.	Total reducing sugars.	Glucose.	Fructose.	Sucrose.	Starch.	Glyco- side- glucose.
Green $\frac{1}{2}$ -full fruit							
A. Pulp. Means of 22 fingers . . .	28.14	0.056	0.024	0.032	0.149	16.65	0.055
Standard deviation of single observation .	0.61	0.014	0.010	0.012	0.023	2.05	0.027
Yellow $\frac{1}{2}$ -full fruit							
B. Pulp. Means of 18 fingers . . .	23.60	9.33	4.62	4.70	1.73	1.31	2.38
Standard deviation of single observation .	1.26	1.01	0.64	0.74	0.56	0.85	0.85
Green $\frac{1}{2}$ -full fruit							
C. Skin. Means of 12 fingers . . .	11.05	0.083	0.040	0.043	0.072	2.362	0.018
Standard deviation of single observation .	0.41	0.015	0.010	0.023	0.014	0.397	0.005
Yellow $\frac{1}{2}$ -full fruit							
D. Skin. Means of 12 fingers . . .	12.27	1.223	0.681	0.541	0.940	0.229	0.320
Standard deviation of single observation .	1.20	0.380	0.247	0.177	0.105	0.101	0.136

The data as given are summarized from the results of a survey of the variation in composition of central and outer fingers of a hand, of the fingers from different hands of a bunch, and of fingers from comparable hands in three bunches; therefore the variability shown is maximal for the single finger sampling method. The values given for the pulp in row A are the means of the results of 22 analyses of central and outer fingers (numbers of fingers of each class analysed shown in brackets in this order) of the 1st (2, 2), 3rd (3, 2), 4th (3, 2), and 9th (2, 0) hands of bunch X, of fingers from the 3rd hand (3, 0) of bunch Y, and of the 4th hand (3, 0) of bunch Z. Row B contains the mean values from 18 analyses of the pulps of ripe fingers both central and outer from the 3rd and 4th hands of the three bunches. Rows C and D each contain the mean values from 12 analyses of the skins of green and ripe fingers respectively; taken from central and outer fingers of the 1st, 3rd, and 4th hands of bunch X only, in row C and from the 3rd and 4th hands of bunches X and Y in row D. The value for the standard deviation of a single observation from the mean provided in each row against the appropriate mean, gives a measure of the variation of the values obtained for the composition of single fruits.

show fair uniformity among analyses of fingers taken from comparable bunches at the same time. Closer agreement may be expected (Wardlaw, Leonard, and Barnell, 1939) if samples are taken from the same hand and from adjacent fingers in the same row of the hand. In the present work sample fingers were always taken from the middle of the third or fourth hand of the bunch.

The bananas used in this study were obtained during the period June to

August 1937 from the Montserrat district of Trinidad. The records fall into two series:

(a) Those based on bunches of known age (which had been tagged at the time of 'shooting', i.e. of emergence of the young bunch at the crown of the plant); these bunches, of ages ranging from 0 to 79 days, were transported, and sampled on the day they were harvested.

(b) Those based on samples taken from plants which were judged to carry standard ' $\frac{3}{4}$ -full' (English grade) fruit on June 18th and which were on that day, from general experience, assigned the arbitrary age of 80<sup>1</sup> days from the day on which they were 'shot' (emerged at the crown).

The ages of bunches in series (b) are given in inverted commas whenever they are referred to in the text. Three plants, A, B, and C were initially selected, but C fell down when aged '106' days. A and B were propped so that the weights of their bunches should not cause the collapse of the stems. To afford protection against birds, bats, &c., the bunches were enclosed in bags.

Series (a) bunches were received at the laboratory within two or three hours of cutting and immediately sampled, divided into skin and pulp, weighed and placed in hot alcohol;<sup>2</sup> in series (b) the samples consisted of fingers removed at intervals from plants A, B, and C; the fruits were transported to the laboratory, in sealed boxes, where they were separated into skin and pulp, weighed, sliced, and placed in hot spirit within two hours of cutting. Samples were then stored to await analysis.

For each analysis the solid matter of the sample was separated from the alcohol extract by sieving through close-meshed perforated zinc. The solid matter was oven-dried at 100° C., cooled and ground in a mill to a fine powder. This powder was placed in a Soxhlet thimble and extracted in a Soxhlet for six hours, using the original alcohol solution as extractant. The extracted residue was dried, weighed, and samples from it used for starch estimation. The alcohol extract was made up to volume and samples used to determine (i) the total alcohol-soluble substances by evaporation to dryness at 100° C., and (ii) the titratable acid. The dry-matter contents of the tissues were obtained from the data on the amounts of total alcohol-soluble substances and extracted residues.

For sugar analysis the alcohol was removed in vacuo and the aqueous solution cleared with basic lead acetate and sodium phosphate. The methods of analysis were as described in earlier work on the carbohydrates of the wheat plant (Barnell, 1936) except that an active and selective invertase preparation<sup>3</sup>

<sup>1</sup> No high degree of precision can be assigned the arbitrary age of 80 days here allotted bunches bearing  $\frac{3}{4}$ -full fruit and, in fact, inspection of the graphs shows that the use of 85-90 days would have given greater continuity of developmental curves in many cases.

<sup>2</sup> No neutralizing agent was added to the spirit to prevent inversion of sucrose as preliminary tests showed that sucrose values were unchanged by storage with excess of calcium carbonate in the alcohol.

<sup>3</sup> 'Convertit' prepared by the Wallerstein Company Inc., New York, and distributed by the Nulomoline Company.

was used for the inversion of sucrose and the non-fermentable fraction of the total taka-diastase hydrolysis products was not systematically determined in the present work. In those samples which were investigated the proportion of non-fermentable products was found to be low in comparison with the fermentable, i.e. starch, fraction. Preliminary investigations have given no evidence of the presence of fructosans (fructose-yielding polysaccharides) in either green or ripe fruit.

All carbohydrate quantities will be expressed in terms of glucose<sup>1</sup> and the data are presented in two ways: firstly, as percentages of the fresh weight of skin or pulp and secondly as total amounts of each substance in skin and pulp per single finger. The expression of data as percentages of the fresh weight of skin or pulp is based on a standard which varies with the water content of the tissues, but this method of expression, provided that data for the percentage dry-matter content of the tissue were also presented, was regarded as most generally satisfactory.

Towards the end of the sampling period in series (b) various ripening changes occurred irregularly in the bunches: data for these fingers, therefore, showed erratic departures from the trends established for green, unripe fruits. Whereas the bunches of certain varieties cultivated in the Orient are said to undergo a uniform normal ripening on the plant, this has not been experienced when Gros Michel plants were kept under observation in Trinidad. The individual fingers coloured, rotted, and fell irregularly throughout the bunch, over a period of time, as from about the '100'th day. In some fingers the skin split, others turned yellowish-green then brown-black and fell off. In plant C sampling was brought to an end by the plant being pulled over by the weight of the bunch, this probably representing the normal fate of plants whose bunches have not been harvested.

To give a generalized impression of the major changes observed during development by smoothed curves, the data obtained have been triple averaged and the calculated observations plotted against appropriately corrected dates, but all relevant original data are available in tabular form in the Appendix.

### III. PERCENTAGE AMOUNTS OF DRY MATTER AND OF VARIOUS CARBOHYDRATES IN THE PULP

#### (a) *Total dry matter, starch, and total sugars.*

The trends of the amounts of total solids, starch, and total sugars expressed as percentages of the fresh weight, in the pulp of the developing fruit are shown in Fig. 1 A. The dry-matter percentage increased consistently from

<sup>1</sup> The conversion from glucose equivalents into actual amounts of fructose and sucrose is given by the following factors: fructose,  $1.031 \times$  glucose equivalent; sucrose,  $1.016 \times$  glucose equivalent.

the day the bunch 'shot' until approximately 60 days later, after which—allowing for the change-over from series (a) to series (b) sampling—there was little change until after '100' days when a steady fall began, i.e. the water content of the pulp began to rise. This change was coincident with and, presumably, in part the result of, increasing soluble sugar content.

The amount of total sugars in the pulp was less than 0.5 per cent. on the day the bunch was 'shot' and fell consistently from that level to a very small amount at '100' days, after which it began to increase, attaining a value of 4.06 per cent. on the '130'th day. During the period of low and decreasing sugar concentration the starch percentage increased in two stages; the first stage of rapid consistent increase being from 0 to 60 days approximately and the second stage, of less rapid increase than the first, from 60 to '100' days. After '100' days the rate of hydrolysis of starch exceeded the rate of condensation of sugars to starch and the percentage amount fell from 19.19 per cent. on the '100'th day to 6.79 per cent. on the 130th day.

(b) *Sucrose, glucose, fructose, and glycosidic-glucose.*

Sucrose was the sugar present in greatest amount in the early stages of fruit development (shown in Fig. 2 A) but fell steadily with increasing size of fruit. From '80' days sucrose fell further till by '100' days it was the sugar present in least quantity; it remained at a very low concentration, less than 0.02 per cent. until the '120'th day, after which it rose, attaining to 0.36 per cent. on the '130'th day. Glucose and fructose were present in less than 0.1 per cent. amount until the '100'th day and tended to decrease in amount from the date the bunch 'shot' (i.e. emerged) until this age. After the '100'th day their amounts rose, ultimately reaching values of 2.095 per cent. and 1.604 per cent. respectively on the '130'th day.

The percentage amount of glycosidic-glucose increased progressively throughout the development of the fruit.

(c) *Titrateable acid.*

The titrateable acid, determined by diluting a portion of the alcohol extract with water and titrating with N/10 sodium hydroxide solution, with phenolphthalein as indicator, was relatively high in the very young fruit, requiring for neutralization 92.0 ml. N/10 sodium hydroxide per 100 gm. of fresh pulp. The acidity fell rapidly up to about 40 days (Fig. 3 A). From this age it fell fairly slowly until '100' days, after which rising values occurred coincident with the increasing sugar concentrations and decreasing starch. As fingers of bunches less than 40 days (approximately) old at harvesting do not show the full colour and other changes associated with the ripening of more mature fruit, a relationship deserving of study may exist between acid content and the initiation of ripening.

#### IV. PERCENTAGE AMOUNTS OF DRY MATTER AND VARIOUS CARBOHYDRATES IN THE SKIN

##### (a) Total dry matter, starch, and total sugars.

In the skin of the developing banana fruit the drifts of the dry matter, starch, and total sugars shown in Fig. 1 B were similar to those in the pulp

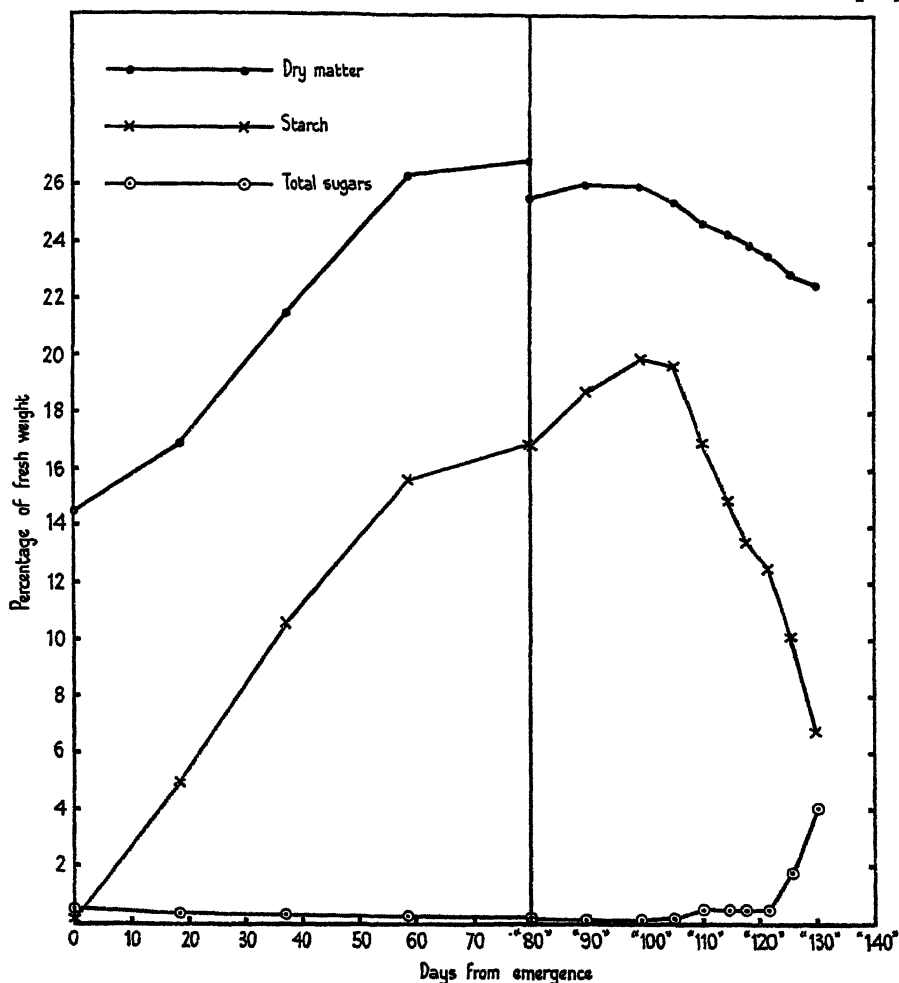


FIG. 1 A. Pulp. Percentage amounts of dry matter, starch, and total sugars from the day the bunches 'shot' till the fingers fell. The vertical line denotes the ' $\frac{3}{4}$ -full' stage of development of the fruit at which the switch-over from series (a) to series (b) samplings took place (see text). Data for the pulp are plotted from the triple-averaged values given in Appendix Table V, derived from the original values of Appendix Tables I and II.

(Fig. 1 A) over the greater part of the period. Total sugars were low at the time of emergence of the bunch (0.133 per cent.) and fell steadily from this



value until approximately the '100'th day after which a rise began, at first slow, but after '120' days relatively rapid, reaching a value of 1.01 per cent. on the '130'th day.

The percentage amount of starch accumulated steadily from a low value,

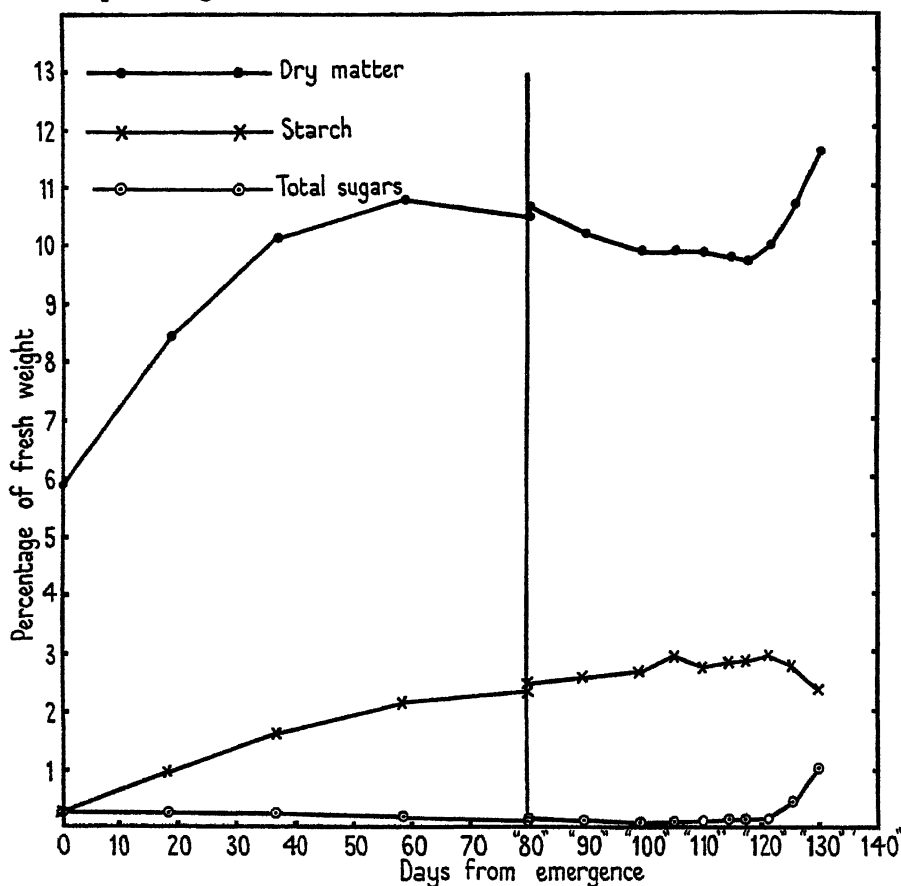


FIG. 1 B. Skin. Description as on Fig. 1 A (Data from Appendix Tables VI, III, and IV)

0.292 per cent., on the day of bunch emergence to 2.96 per cent. on the '121'st day, then falling somewhat to 2.36 per cent. on the '130'th day coincident with the major rise of total sugars.

The percentage dry matter in the skin was relatively low throughout the development of the fruit (Fig. 1 B), it amounted to only 5.89 per cent. at the time of bunch emergence, increasing to 10.82 per cent. on the 58½th day (corrected date); by the 117th day it had fallen to 9.69 per cent., but afterwards rose quickly to 11.62 per cent. on the '130'th day.

(b) *Sucrose, glucose, fructose, and glycosidic-glucose.*

Glucose was to a small extent the dominant sugar in the skin of the young

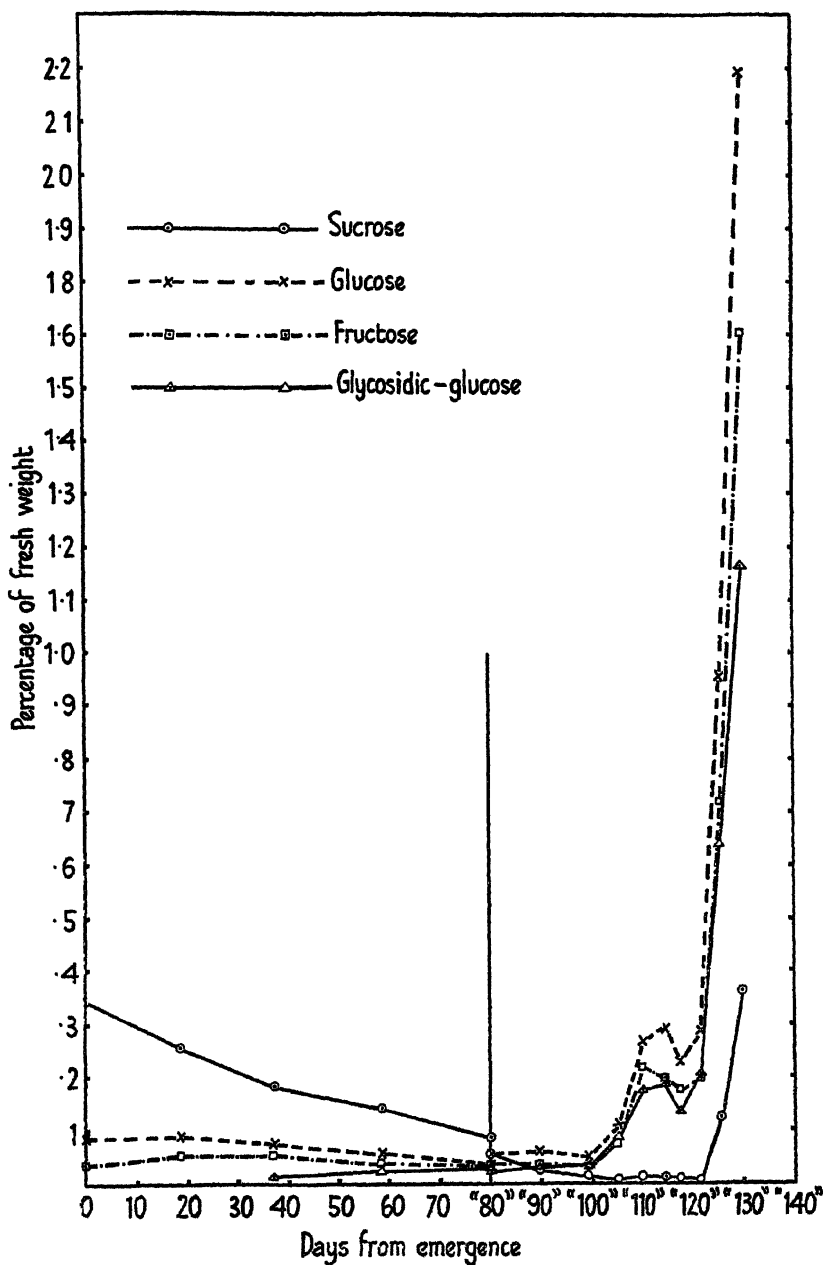


FIG. 2 A. Pulp. Percentage amounts of sucrose, glucose, fructose, and glycosidic-glucose. The vertical line denotes the '1/2-full' stage of development of the fruit at which the switch-over from series (a) to series (b) samplings took place. Points for the pulp have been plotted from the triple-average values given in Appendix Table V, derived from the original values of Appendix Tables I and II.

fruit in the early stages of development (Fig. 2 B), falling in percentage amount steadily till the '105'th day, after which a rise was initiated and continued till the last sampling date, the '130'th day. Sucrose was present in next largest concentration in the skin and followed a similar course to that of

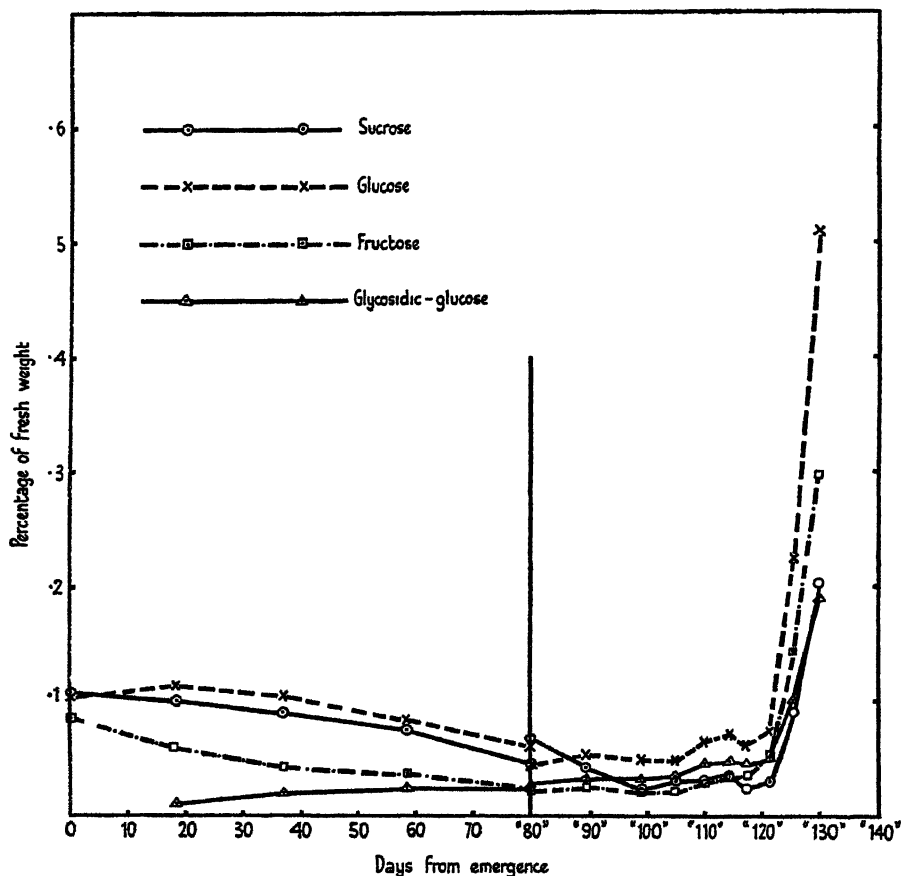


FIG. 2 B. Skin. Description as for Fig. 2 B. (Data from Appendix Tables VI, III, and IV.)

glucose, but fell to its lowest value on the '100'th day, afterwards rising, though with fluctuations, to the 130th day. Fructose was present in approximately half the glucose concentration and followed a similar drift to that of glucose.

The 'bound' or glycosidic-glucose followed a different course from those of the free soluble sugars in that it rose consistently from the first sampling date on which it was estimated until the last sampling date, which suggests that, possibly, substances with the glycosidic linkage were being steadily laid down in localized parts of the tissue rather than uniformly through the tissue.

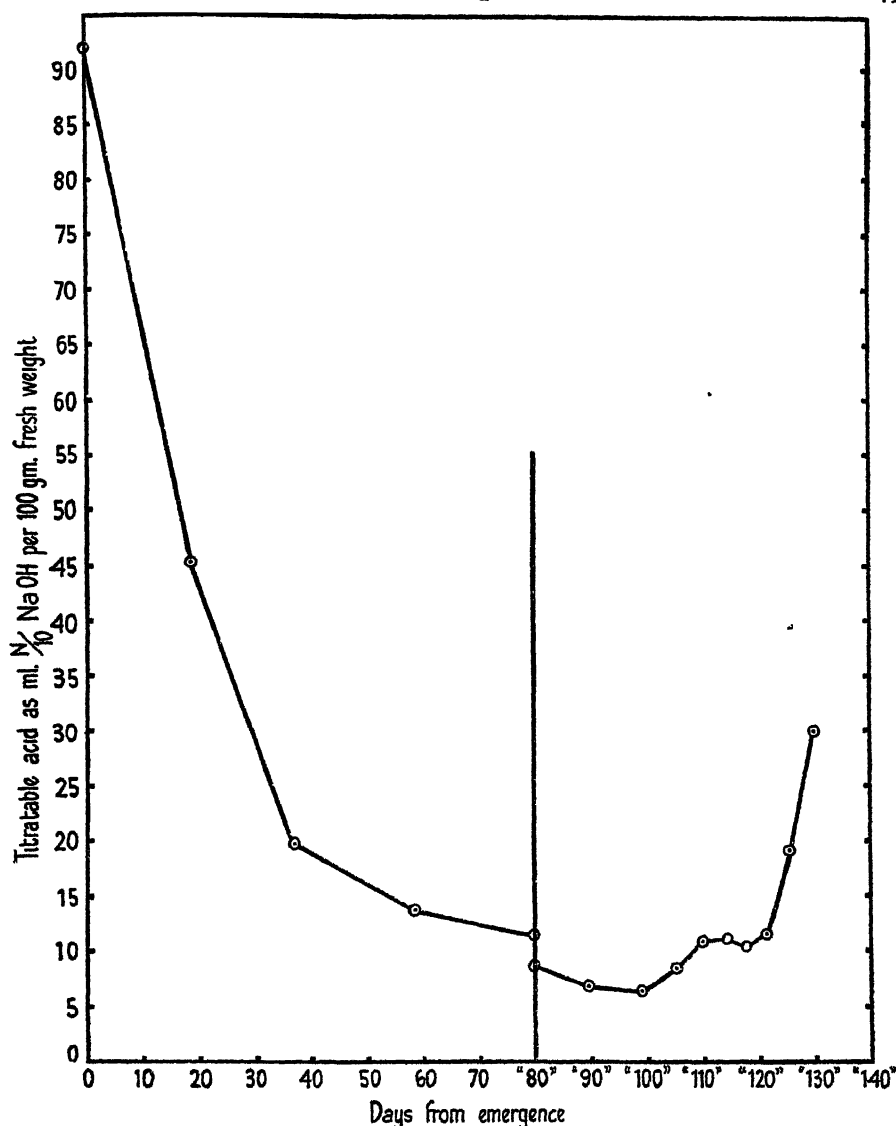


FIG. 3 A. Pulp. Titratable acid expressed as ml. of N/10 NaOH per 100 gm. fresh weight. The vertical line denotes the '¾-full' stage at which the sampling changed from series (a) to series (b). Data for the pulp are from the triple-average values given in Appendix Table V, derived from the data of Appendix Tables I and II

(c) *Titratable acid.*

The percentage amount of titratable acid in the skin was much less than in the pulp of the young fruit, but whereas in the pulp the acid fell in amount to the '100'th day (Fig. 3 A), it rose in the skin from the first sampling date on

which it was estimated until the '100'th day (Fig. 3 B), apart from the break at the transition from series (a) to series (b) data. By the '100'th day the acid in the skin was present in greater percentage amount than in the pulp.

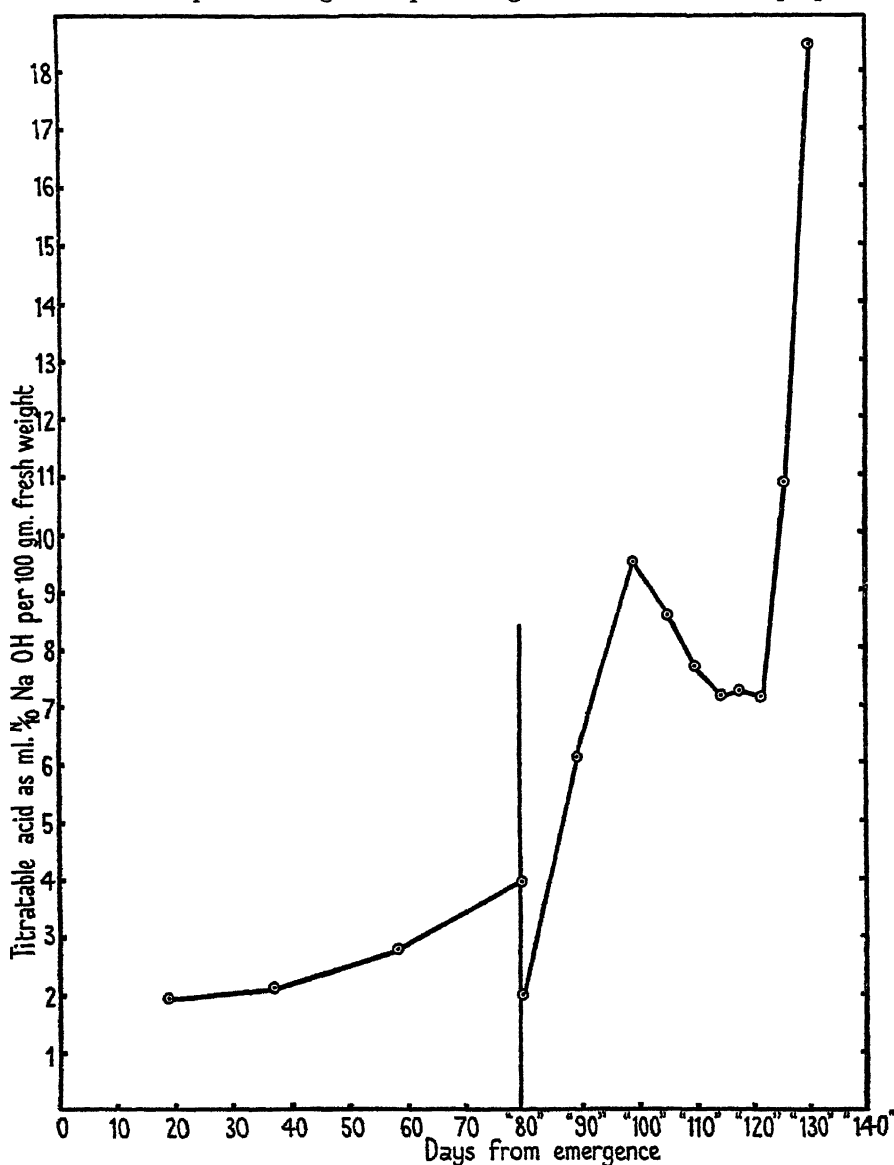


FIG. 3 B. Skin. Description as for Fig. 3 A. (Data from Appendix Tables VI, III, and IV.)

The fall in acid in the skin after the '100'-day stage coincides approximately with the initiation of important metabolic changes. The significance of the fall in acid content is not clear, it is the reverse of what happens in the pulp.

V. PERCENTAGE AMOUNTS OF TOTAL ALCOHOL-SOLUBLE SUBSTANCES,  
SUGARS AND NON-SUGARS IN PULP AND SKIN

Archbold (1938) has indicated the probable importance of that fraction of total alcohol-soluble substances which is not accounted for by the substances estimated, particularly as the amounts of this fraction may follow a similar course to that of the sugars, as in the wheat plant (Barnell, 1936, 1938). In addition to the soluble sugars, the alcohol extract contains fatty substances, glycosides including tannins, nitrogenous substances, organic acids, and inorganic compounds. In the present work the non-sugar fraction may contain all these other non-sugar compounds of which glycosidic-glucose and total acidity only were separately estimated.

Values for the total alcohol-soluble substances, total sugars, and total non-sugars in pulp and skin of the developing banana are given in Table II against the age (in days from bunch emergence) of the fruit.

TABLE II

*Total Alcohol-soluble Substances, Total Sugars, and Non-sugars in Pulp and Skin expressed as Percentages of the Fresh Weight*

Days from emergence.	Pulp.			Skin.		
	Total alcohol-soluble substances.	Total sugars.	Non-sugars.	Total alcohol-soluble substances.	Total sugars.	Non-sugars.
0	6.85	0.463	6.36	0.76	0.293	0.47
16	2.94	0.387	2.55	1.43	0.265	1.16
37	1.76	0.322	1.44	1.47	0.257	1.21
58	1.81	0.229	1.58	1.26	0.186	1.07
80	1.38	0.161	1.22	1.27	0.130	1.14
'80'	0.90	0.152	1.75	1.13	0.133	1.00
'92'	0.97	0.162	0.81	1.24	0.124	1.12
'99'	0.79	0.064	0.73	1.29	0.104	1.19
'106'	0.86	0.059	0.80	1.30	0.054	1.25
'111'	1.48	0.446	1.03	1.25	0.143	1.11
'114'	2.03	0.995	1.03	1.27	0.179	1.09
'118'	0.88	0.130	0.74	1.01	0.103	0.91
'121'	1.01	0.110	0.90	1.10	0.082	1.02
'125'	2.71	1.226	1.48	1.47	0.281	1.19
'130'	6.96	4.060	2.90	2.82	1.010	1.81

The first five rows of the table are from the series (a) samples and the remaining rows from the series (b) samples. The values in the first row are the means of two observations and the remainder of the series (a) values are means of four observations. All series (b) values are the means of two observations from plants A and B respectively. The non-sugar values given in column 4 for the pulp and in column 7 for the skin were obtained by difference from the observed values of total alcohol-soluble substances and the total sugars (sucrose, glucose, and fructose).

It will be observed that the amounts of the non-sugar fraction are very high relative to the sugar fraction, and that although the drift of the amount of non-sugars is approximately similar to that of the sugars the relation between them is very variable. This may be due to incomplete extraction of non-sugars as the extraction was standardized for sugars only. The large amounts of the non-sugar fraction in addition to the glycosides, which have been partially examined, and the fact that this fraction apparently has a well-defined trend during development in both pulp and skin, suggest that its constituents, particularly fats and organic acids, are worthy of further investigation.

#### VI. TOTAL AMOUNTS OF DRY MATTER AND OF VARIOUS CARBOHYDRATES IN THE PULP AT SUCCESSIVE STAGES OF DEVELOPMENT

The quantities of different carbohydrates in the skin and pulp of a single finger during development of the fruit on the plant are plotted in Figs. 5 A, B; 6 A, B, and 7 A, B; and in Fig. 4 the fresh weights for the corresponding chronological ages of fruits are given.

This presentation of the data permits a ready survey of the chemical development of the fruit. It also emphasizes the stage at which the change occurs from the accumulation of carbohydrates, mainly in the form of the storage or reserve form of starch, to the stage of incipient ripening, where hydrolysis of starch and accumulation of sugars are the major processes.

In the absence of data on respiration rates of fruits developing on the plant, and also in view of the somewhat inadequate sampling technique, no detailed discussion will be attempted of the quantitative interrelations of starch and sugars at this stage of incipient ripening, nor of the total translocation of carbohydrates from the plant to the finger and its distribution there between skin and pulp. The data submitted are intended to serve as a broad survey of some aspects of the metabolic development of the fruit on the tree; for closer analysis a more detailed study will be required.

##### (a) *Total dry matter, starch, and total sugars.*

The dry-matter accumulation followed a typical sigmoid curve of development apart from the break at the transition between the two sampling methods (Fig. 5 A), the rate being greatest between '80' and '110' days, i.e. during the period *immediately following* the stage at which the fruit is harvested for the English market. Whether or not this increased growth rate during the later stages in development is of general occurrence could be determined only by several repetitions of this investigation. The rate of accumulation decreased after '110' days when starch hydrolysis and sugar accumulation were in progress and, presumably, the respiration rate of the fruit was increasing.

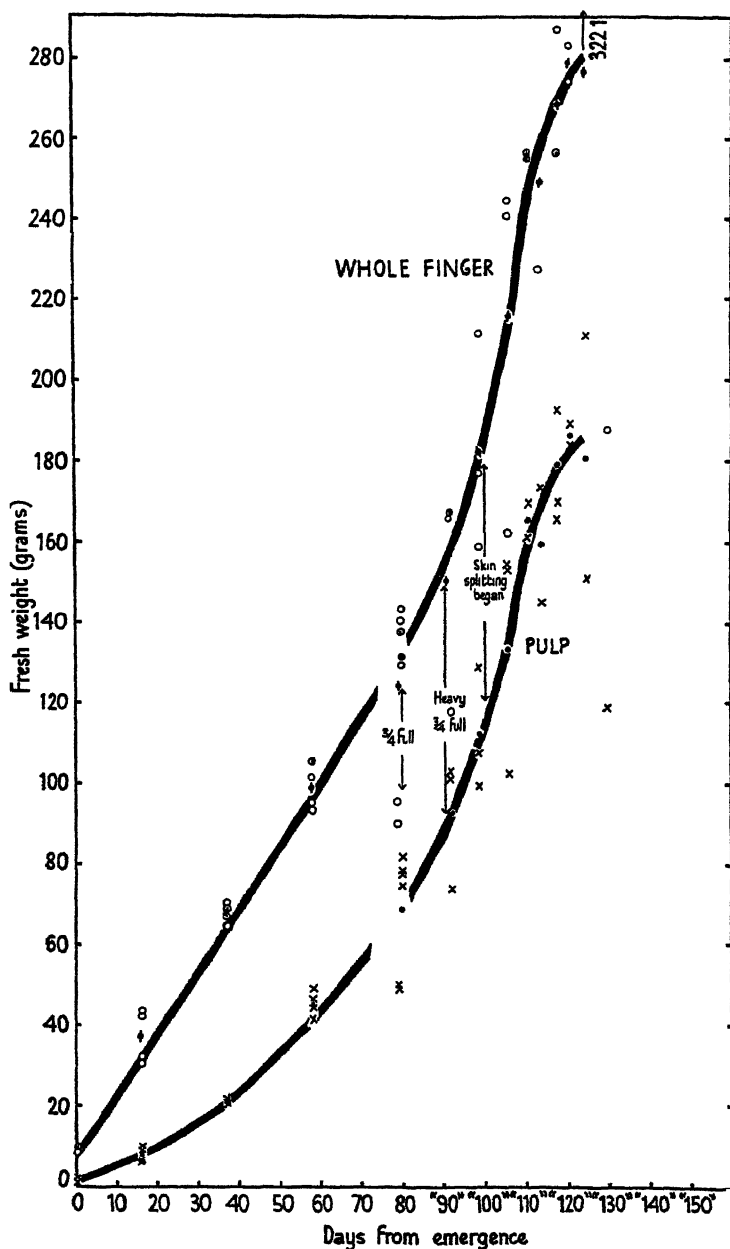


FIG. 4. The growth of the whole finger and of the pulp of the banana on the plant. Values for the fresh weights of whole fingers are shown by circles and for pulp by crosses. Approximations to smooth curves were obtained by plotting the means of the values obtained for each sampling date (whole-finger-weight means indicated by dots with vertical lines drawn through them and pulp-weight means by dots); through these points smooth curves were drawn by eye.



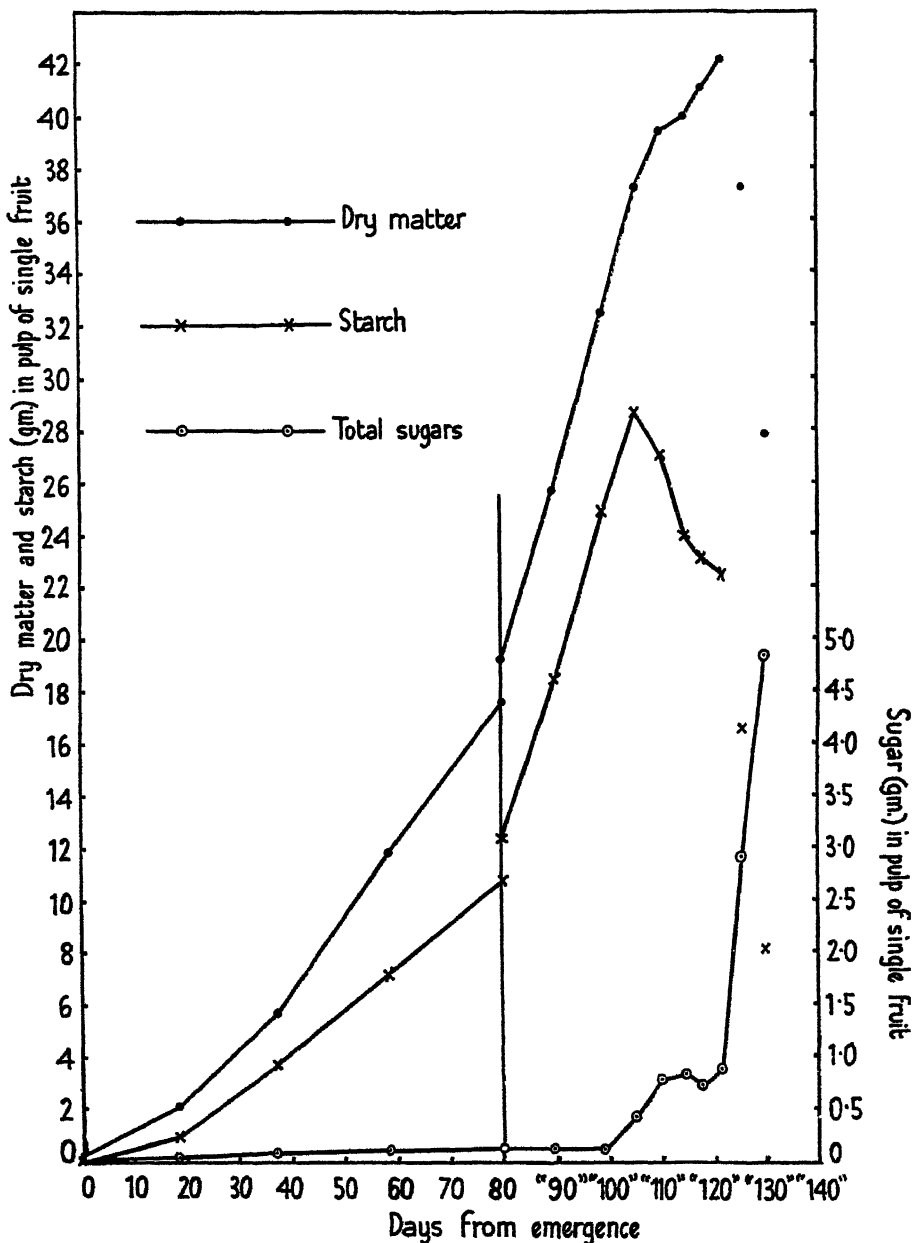


FIG. 5 A. Pulp. Dry matter, starch, and total sugar of pulp expressed as gm. per single fruit. The vertical line indicates the attainment of the '¾-full' stage of development by the fruit and also the change from series (a) to series (b) sampling methods. The last two values for total dry matter and for starch have not been joined to their respective curves as the small size of the residual single finger available for sampling rendered these values unrepresentative in comparison with previously sampled fingers; on the same count the final two values for total sugars must be regarded as low.

Data for the pulp were plotted from the triple-averaged values of Appendix Table VII.

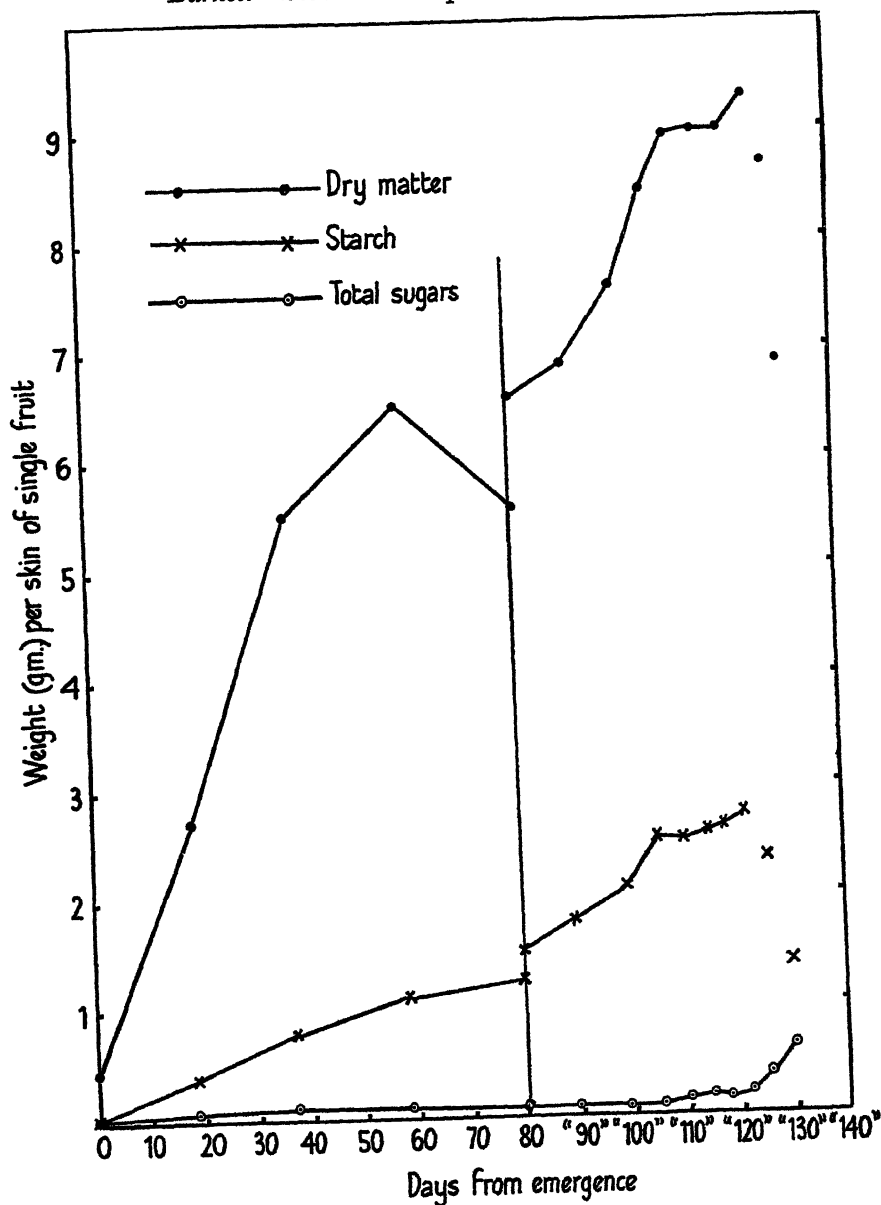


FIG. 5 B. Skin. Description as for Fig. 5 A. (Data from Appendix Table VIII.)

Starch accumulated at first slowly, then after 20 days more rapidly, and, after a break at the sampling transition, most rapidly after '80' days, reaching a peak value after '105' days. From the '105'th day the amount of starch in the pulp decreased, falling to approximately 8 gm. in the sample taken on the

'130'th day. But since by this date most of the fruits on the bunch had rotted or fallen off and the fruit sampled was undersized it cannot be considered representative.

Total sugars accumulated but slowly between 0 and '100' days and then rose, somewhat irregularly, to the last sampling date.

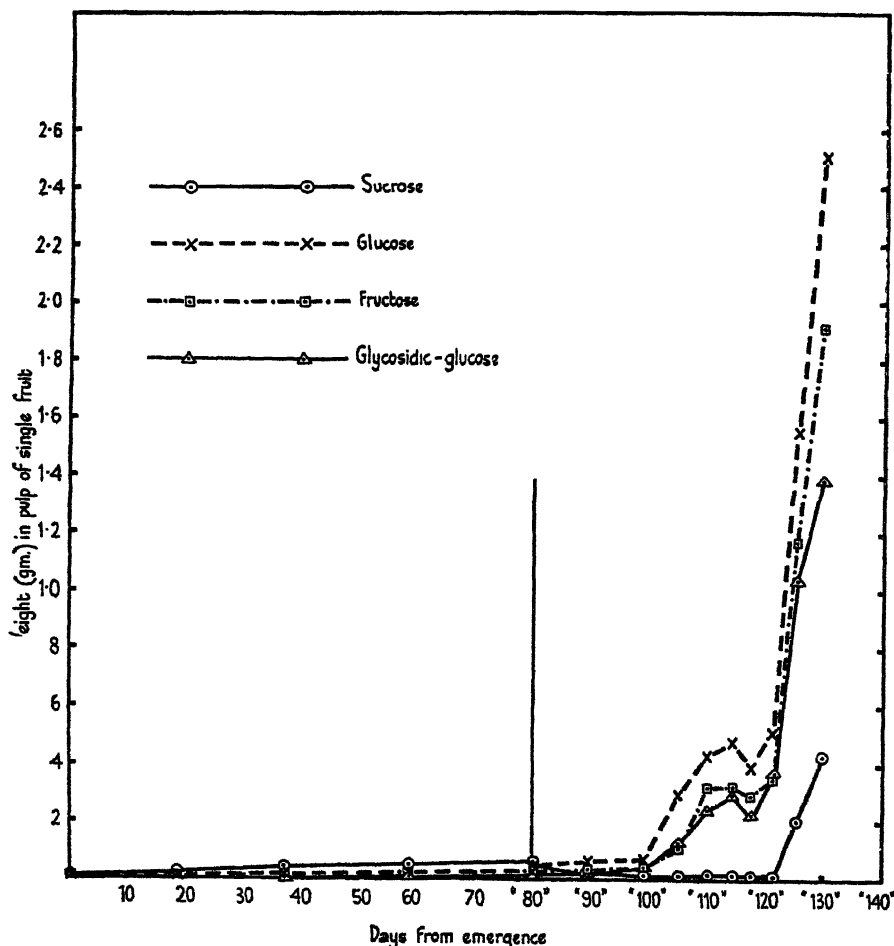


FIG. 6 A. Pulp. Sucrose, glucose, fructose, and glycosidic-glucose as gm. per single fruit. The values for glycosidic-glucose in the pulp up till 80 days were not triple-averaged. The vertical line indicates the '3/4-full' stage of development and also the change from series (a) to series (b) sampling. Points for the pulp were plotted from the triple-averaged values obtained from Appendix Table VII.

(b) *Sucrose, glucose, fructose, and glycosidic-glucose.*

There was little accumulation of any sugar until '100' days (Fig. 6 A). Till 80 days sucrose was present in greatest amount in the pulp showing a slight

increase, whereas it represents a decreasing percentage (Fig. 2 A), but from '80' days the amount fell till '120' days, after which a small rise occurred, continuing to the last sampling date. Glucose and fructose showed little increase up to '100' days and then rose in amount, with glucose invariably

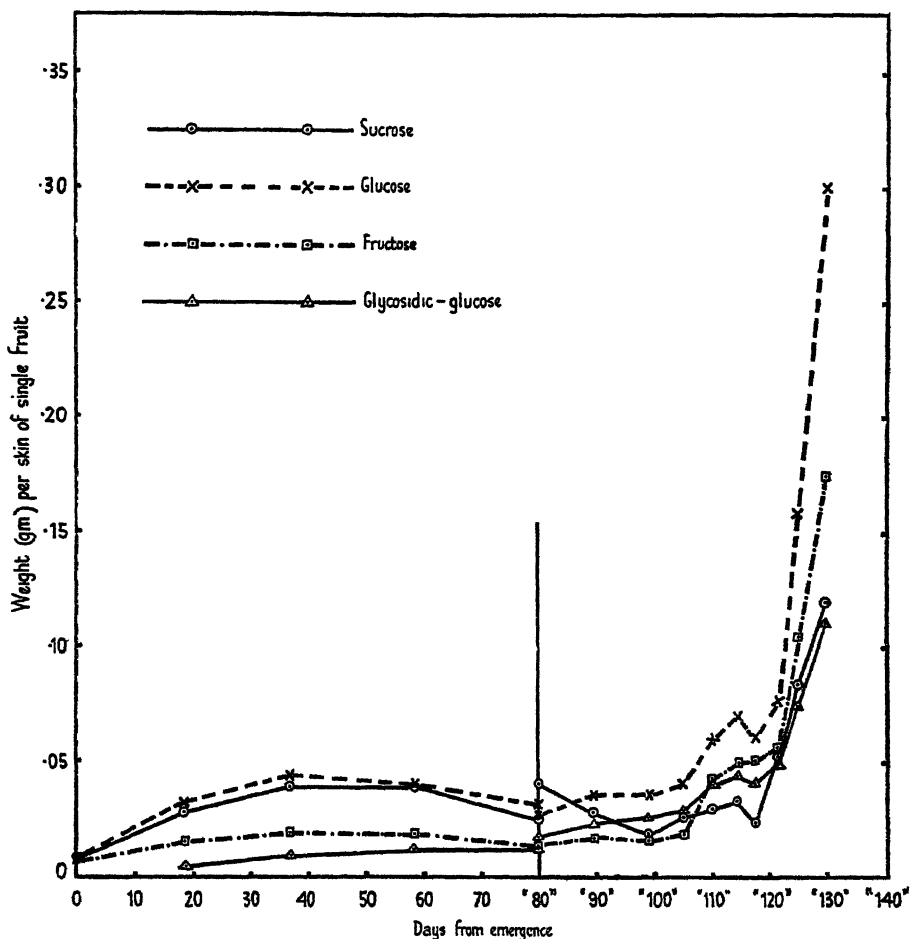


FIG. 6 B. Skin. Description as for Fig. 6 A. (Data from Appendix Table VIII.)

in excess of the fructose. The accumulation of glycosidic-glucose was also very small until after '100' days when it increased coincidentally with the free hexoses.

### (c) Titratable acid.

The accumulation of titratable acid in the pulp, expressed as ml. N/10 NaOH per single finger, is shown in Fig. 7 A. The acid increased steadily in amount, apart from the sampling transition break at 80 days, until '100' days, and then

the amount present increased rapidly. The accumulation rate of acids during the first '100' days did not keep pace with that of the total fresh weight of the fruit, accordingly the percentage amount of titratable acid fell over this period (Fig. 3 A).

## VII. TOTAL AMOUNTS OF DRY MATTER AND OF VARIOUS CARBOHYDRATES IN THE SKIN AT SUCCESSIVE STAGES OF DEVELOPMENT

### (a) *Total dry matter, starch, and total sugars.*

The accumulation of dry matter in the skin, Fig. 5 B, appeared to proceed in three phases. The first phase lasting from 0 to approximately 40 days was one of relatively rapid accumulation; this was followed by a phase lasting from approximately 40 to approximately '90' days during which the rate of accumulation was considerably slowed down. The third and last phase lasting from '90' days to the end (apart from the values derived from the final, unrepresentative, sampling) was again one of relatively rapid accumulation. This trend contrasts with the sigmoid curve of dry-matter accumulation in the pulp (Fig. 5 A). The second phase is the one during which fruit of the various required grades would be cut for export and the third phase is one in which alterations occur in the relation of skin to pulp, expansion frequently resulting in splitting of the skin. The relation between the anatomical development of the fruit and these biochemical phases is under investigation and accordingly will not be further discussed here.

The progress of starch accumulation in the skin is also shown in Fig. 5 B. It was present in negligible amount on the date the bunches were shot, but increased fairly steadily throughout the period from 0 to '120' days, falling in amount somewhat to the last sampling date on the '130'th day.

Total sugars were present in very small quantity from the appearance of the bunch until approximately the '110'th day when a slow accumulation began, continuing until the last sampling date. Thus it would appear that sugars entering the skin during development of the fruit are rapidly condensed to starch and other stable carbohydrate-containing compounds, the concentration of sugars remaining very low until ripening begins. From the curve of percentage composition shown in Fig. 1 B it would seem that the critical values of concentration of the individual sugars for condensation slowly decrease through the developmental period until incipient ripening intervenes.

### (b) *Sucrose, glucose, fructose, and glycosidic-glucose.*

The accumulation curve for total sugars shown in Fig. 5 B is analysed into its component sugars, sucrose, glucose, and fructose in Fig. 6 B and their accumulation depicted on a larger scale; the accumulation of the 'bound' or glycosidic-glucose is also shown.

Glycosidic-glucose increased in amount over the entire period, slowly until 80 days, then rather more rapidly from '80' to '105' days, and after that, with some fluctuation, more rapidly up to the last sampling date. But the final amount accumulated was not large, 0.111 gm. only.

Glucose, sucrose, and fructose all increased in amount up to the 37th day but then fell slightly to the 80th day. Glucose was present in slightly the greatest amount all through the developmental period, increasing after the 80th day, at first, fairly slowly, to the '118'th day and then, relatively quickly, to the last sampling date. Sucrose was the predominant sugar during the early stages of development of the pulp (Fig. 6 A). Sucrose in the skin decreased in amount from the 80th day, no appreciable increase occurring until after '115' days. Fructose showed no appreciable increase or decrease in time compared with glucose, remaining consistently lower in amount.

(c) *Titrateable acid.*

The sequence of the total amounts of titrateable acid found in the skin of the banana is set out in Fig. 7 B. The general drift is similar to that in the pulp (Fig. 7 A) though the actual amounts are considerably lower (about one-quarter). Accumulation was slow until after '80' days, the rapid accumulation rate which succeeded that date was temporarily arrested between '105' and '120' days, and then continued to the final sampling date. The significance of this arrest, which is practically synchronous with a similar one in the pulp acid-accumulation curve (Fig. 7 A), is not clear but is apparently associated with incipient ripening. It is desirable that work should be undertaken on the pH of the skin and pulp tissues as opposed to direct estimates of titrateable acid.

#### VIII. ACCUMULATION RATES OF CARBOHYDRATES IN PULP AND SKIN IN RELATION TO EXPORT GRADES

It is possible to differentiate the various grades to some extent by the daily rates of accumulation of dry matter, starch, and total sugars, and for this purpose the growth period of the banana fruit has been divided into four arbitrary phases. Phase (1) includes the period from the time of 'shooting' of the bunch up to the development of the  $\frac{3}{4}$ -full fruit, phase (2) from the  $\frac{3}{4}$ -full stage to the heavy  $\frac{3}{4}$ -full, phase (3) from the heavy  $\frac{3}{4}$ -full stage to '100' days, when splitting of the skin and other symptoms of incipient ripening appeared, and phase (4) from '100' days to the last practicable observation on the '120'th day.

In Table III the mean daily rates of accumulation of dry matter, starch, and total sugars in both pulp and skin are set out for each of these phases. It will be seen that bananas are usually cut (phase (2)) during the stage of rapid dry matter (consisting almost entirely of starch) accumulation in the

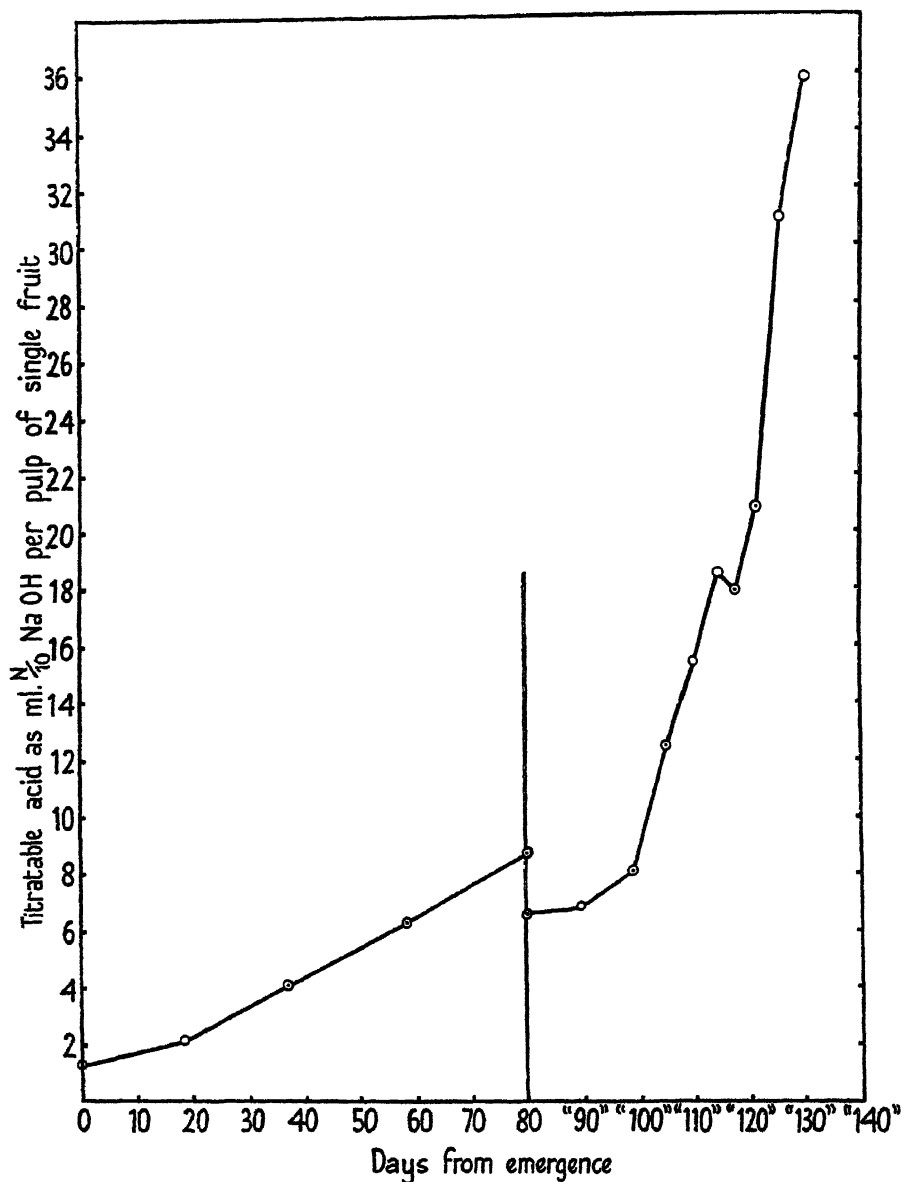


FIG. 7 A. Pulp. Titratable acid expressed as ml. N/10 NaOH per 100 gm. fresh weight. The vertical line indicates the '¾-full' stage of development of the fruit and the change in sampling from series (a) to series (b). The values plotted for the pulp are triple-averaged, as given in Appendix Table VII.

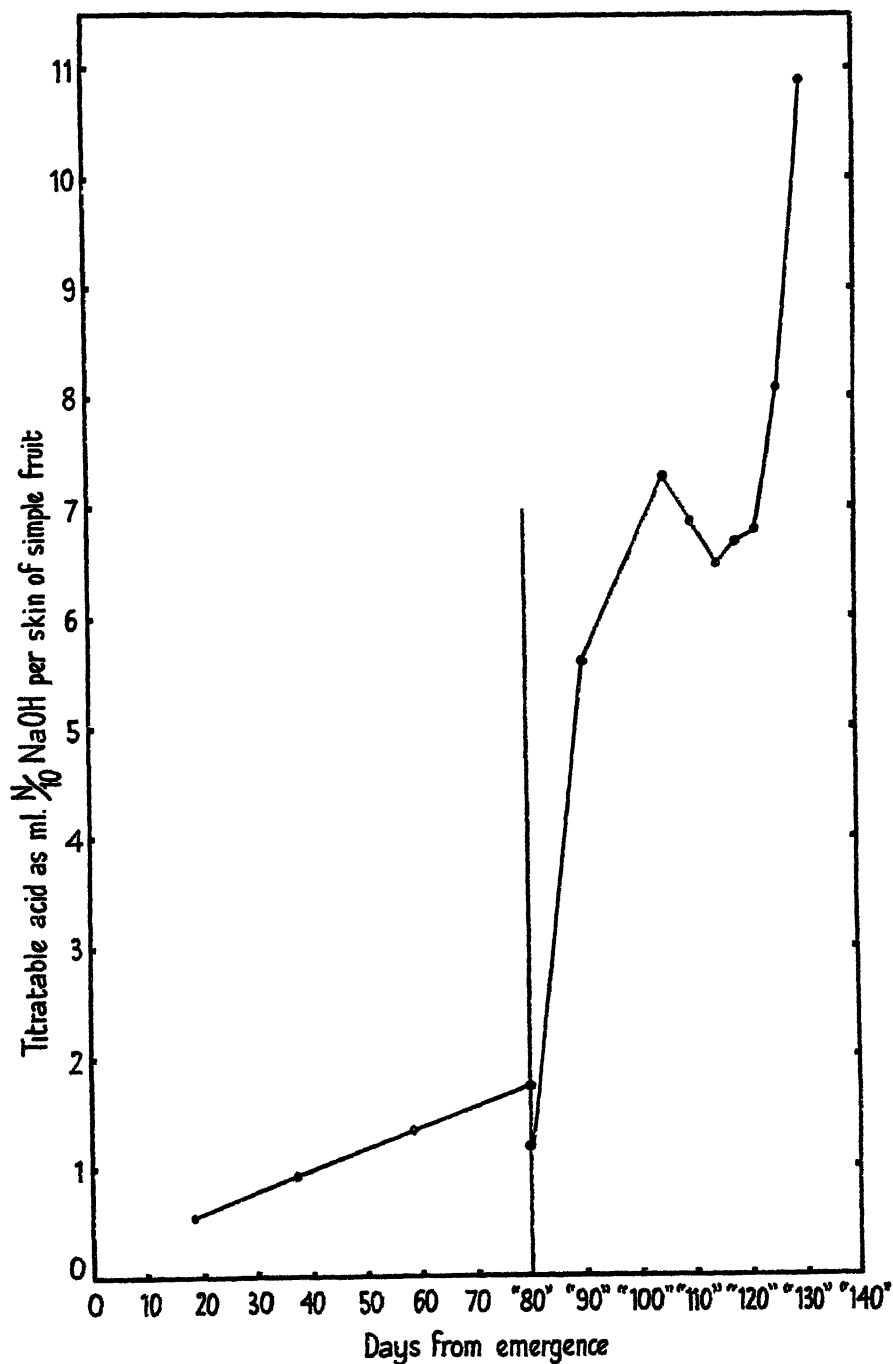


FIG. 7 B. Skin. Description as for Fig. 7 A. (Data from Appendix Table VIII.)



pulp and a comparison of the values in columns 3 and 4 shows that the mean daily rate of accumulation was higher in the ten days succeeding the usual stage of cutting the heaviest grade for export than in either the period between the  $\frac{3}{4}$ -full and the heavy  $\frac{3}{4}$ -full grades or the period from the shooting of the bunch to the  $\frac{3}{4}$ -full grade. Total sugars accumulated in the pulp at a very slow rate during the first three phases while, in the skin, during the second and third phases decreasing amounts of total sugar were observed.

TABLE III

*Mean Daily Rates of Accumulation of Total Dry Matter, Starch and Total, Sugars, in gm. per Finger, during the Phases of Growth of the Banana Fruit*

Substance.	Pulp				Skin			
	1st phase from 'shooting' to $\frac{3}{4}$ -full (80 days)	2nd phase $\frac{3}{4}$ - to heavy $\frac{3}{4}$ -full (10 days)	3rd phase heavy $\frac{3}{4}$ -full to '100' days (10 days)	4th phase '100' days to '120' days (20 days)	1st phase from 'shooting' to $\frac{3}{4}$ -full (80 days)	2nd phase $\frac{3}{4}$ - to heavy $\frac{3}{4}$ -full (10 days)	3rd phase heavy $\frac{3}{4}$ -full to '100' days (10 days)	4th phase '100' days to '120' days (20 days)
Total dry matter	0.217	0.675	0.738	0.419	0.064	0.031	0.087	0.062
Starch	0.135	0.624	0.684	—0.242	0.015	0.026	0.036	0.027
Total sugars	0.001	0.001	0.004	0.033	0.001	—0.001	—0.001	0.005

The values of the daily accumulation rates given for the pulp and skin of the banana finger during development have been obtained by inspection from Fig. 4 and Fig. 7 A in which the data of Appendix Tables VII and VIII were plotted. During the first three phases of development given for both pulp and skin starch was increasing in amount while in the fourth phase hydrolysis had begun. The rate of accumulation of total sugars was very low in the pulp until the fourth phase, while in the skin the total amount was decreasing during the second and third phases, accumulation beginning again in the fourth phase.

Some other fruits besides the banana are harvested before they are full grown and have attained their maximum carbohydrate content. Harvesting of pears and of freestone varieties of peaches in an immature stage of growth is carried on in the State of California for early shipment, resulting in considerable sacrifice of yield, Allen (1932). The loss is in respect both of total weight of fruit and carbohydrate content, in these instances sugars, which materially increase in fruit left on the tree.

### VIII. DISCUSSION

The banana differs from other fruits in that cut from the tree at any except the very early<sup>1</sup> stages of its development it will ripen to an edible condition. That the developmental stage at harvesting influences, among other factors, the length of time required for subsequent ripening, and hence the possible storage life, has long been known. No quantitative data, however, have been

<sup>1</sup> Less than 40 days.

available as to the size which a fruit could attain if left on the plant; information on the biochemical development of the fruit was also lacking.

Accordingly, the first approach to the biochemical problems of the banana in storage has been to study its development on the plant, partly to determine the upper size limit at which no evidence of ripening is observable and at which cutting for storage may therefore still be practicable, and partly to define the composition of the present standard grades. The final quality of fruit left to ripen on the plant was also envisaged as an interesting and useful investigation, but, as already explained, the fruit under observation did not ripen to an edible state but split, fell off, and became rotten. Yellowing fruits observed in plantations, in fact, are usually the result of disease or injury.

During the development of the banana, sugars remain at very low concentrations in both the pulp and skin and starch is accumulated, though to a much greater extent in the former than the latter. It is of interest to contrast this starch accumulation and low sugar content with the apple, Archbold and Widdowson (1931), in which no starch accumulates during the first three weeks of growth and in which the main carbohydrates stored in fruit on the tree are fructose, sucrose, and to a lesser extent glucose. A noteworthy feature of the present investigation is that the rate of accumulation of starch and of total dry matter during the two or three weeks following the age-level at which the fruit would be cut for export to England considerably exceeded that of the weeks preceding this.

The composition of the various grades of fruit as described by their percentage carbohydrate contents does not show a clear relation to their maturity. Reference to Figs. 1 A to 3 B inclusive will show that there is a small difference in the percentage composition of  $\frac{3}{4}$ -full fruit, heavy  $\frac{3}{4}$ -full, and the fruit of the following phase from heavy  $\frac{3}{4}$ -full to '100' days' growth.

With age the fruit increased in size and in weight, that is, in total content of dry matter and water. Since decreasing storage life is associated with increasing grade of fruit it follows that, for fruit from a particular district,<sup>1</sup> maturity must be defined rather by the size of the fruit and its total content of starch, sugars, water, &c., than by its percentage composition. As shown in an earlier section it is possible to differentiate the various grades to some extent by the daily rates of accumulation of dry matter, starch, and total sugars during arbitrarily selected phases of the growth period of the banana fruit.

About three or four weeks before the banana finger had attained its maximum size (i.e. after approximately 125 days from emergence, see Fig. 4) indications of incipient ripening appeared in the carbohydrate composition of the pulp. After '100' days the starch percentage was observed to decrease and simultaneously the concentrations of the soluble sugars to increase.

<sup>1</sup> It is necessary to emphasize that a gradation by size can only be applied to a restricted locality as it is well known the size of fruits corresponding to the same storage grades differ widely, e.g. between the fruits grown on the fertile alluvial plains of Central America and those grown in the West Indies.

Examination of the accumulation data for the pulp during this period (Fig. 5 A) showed that the amount of starch hydrolysed exceeded the increase in amount of soluble sugars suggesting, amongst other possible explanations, a high rate of carbon loss in respiration. At this stage, the skin was subjected to mechanical strain by the expansion in volume of the pulp, and splitting was of frequent occurrence. The expansion of the pulp appeared to be due to absorption of water consequent on the increasing concentrations of soluble sugars; Fig. 1 A shows decreasing values for the percentage amount of total dry matter in the pulp after '100' days while the total weight was still increasing, i.e. the water content was increasing. In the skin the indications of incipient ripening were delayed until approximately '120' days, after which soluble sugars increased and starch decreased in percentage amount. The dry matter or water content (Fig. 1 B) of the skin showed little change from '100' to '120' days, the water loss occurring simultaneously with the carbohydrate evidence of incipient ripening.

It is apparent that these ripening changes, as indicated by carbohydrate composition, began in the pulp, and that the skin was not sufficiently elastic to permit of the swelling of the pulp due to water intake (from the bunch stem) and so split. Data will be presented in a later communication confirming that, as already shown by Gore (1914) and Smith (1932), in fruit ripened off the tree water is withdrawn by the pulp from the skin during the process.

There was no initial rise in the acidity of the pulp as observed in the developing apple (Archbold, 1932), the grape (Copeman, 1927), and in the mango (Wardlaw and Leonard, 1936), but, simultaneous with the synthesis of starch (cf. Archbold, 1932) a continuous fall occurred throughout development until starch hydrolysis began (after the '100th' day) when rising values for the acid content were obtained. Data have been obtained showing that rising acidity in the pulp invariably occurs during the ripening of bananas whether held at tropical temperatures or in ripening rooms at 68° F. after a period of refrigerated storage. The skin of the banana approximated rather more closely in its acid behaviour with other fruits. There was a gradual increase in acidity until '100' days and then falling values were obtained succeeded, however, after three samples by a new, continued, rise, so that in the skin as well as in the pulp increasing acid values were associated with ripening. In peaches, Nightingale, Addoms, and Blake (1930), rising acid values occurred during development with a sudden fall on their becoming eating ripe; it will be shown in later publications that falling acidity in the pulp and to a less extent in the skin of the banana is characteristic of the attainment of the 'over-ripe' stage.

A characteristic feature of the later stages of the ripening of detached fruits is the large loss of water from the skin. The beginning of this process is observable in Fig. 1 B, where an increase of approximately 2 per cent. in the dry-matter content is shown between the '118th' and '130th' days. Thus although complete ripening of Gros Michel fruit did not take place when left

on the plant, incipient ripening occurred after the bunch had been 'shot' for approximately '100' days, i.e. when the fresh weight of whole fingers was approximately 50 per cent. greater, and of pulps approximately 60 per cent. greater, than in fruit harvested for the English market.

## IX. SUMMARY

1. The changes in dry matter, starch, sucrose, glucose, fructose, glycosidic-glucose, and acidity in the pulp and skin of the banana fruit during development have been followed from the time the bunch emerged until the fruits fell or rotted.

2. The initial amount of soluble sugars in the pulp tissue was low ( $<0.5$  per cent.) and fell to a negligible amount by '100' days; during this period the starch content steadily increased; total dry-matter percentage increased for approximately 60 days, thereafter increasing only slightly until '100' days. After '100' days the percentage amount of starch in the pulp decreased, total sugars increased, and the water content simultaneously rose. The drifts of the percentage amounts of the sugars; sucrose, glucose, fructose, glycosidic-glucose, and also of titratable acid have been followed and their interrelations discussed.

3. Total sugars in the skin, initially present in low concentration, decreased until the rooth day, after which increasing values were obtained. Starch increased continuously until the '121'st day, falling values being then obtained. The dry-matter content of the skins rose from an initial low value until approximately 60 days, falling somewhat after this, but subsequently rising again. Analytical data, similar to those for the pulp, are submitted.

4. In view of the proportionately large amounts of alcohol-soluble substances, as compared with sugars, the need for further investigation of the constituents of this fraction is stressed.

5. The data are also represented in the form of accumulation curves, so that the amounts, per single fruit, of each estimated substance in pulp and skin, can be followed stage by stage during development. They provide a basis for further biochemical investigations of fruit during storage and ripening when harvested at different levels of development.

6. Until the arbitrary '100' day age level the clearly defined drifts of carbohydrate, &c., are of a developmental nature; thereafter a difference in each trend appears, usually first seen in the pulp, but closely followed in the skin. These trends constitute the biochemical expression of ripening of fruit still attached to the plant.

7. The ripening process in uninjured fruit left on the plant usually attains the incipient stage only, a small accumulation of sugars and relatively little starch hydrolysis taking place. The earliest indication of incipient ripening is seen in the splitting of the skins; this occurs irregularly through the bunch after '100' days.

## APPENDIX TABLE I

*Development on the Plant: Series (a). Composition of Pulp expressed as Percentages of Fresh Weight*

No.	Days from emergence.	Total dry matter.	Total reducing sugars.	Glucose.	Fructose.	Sucrose.	Starch.	Glyco-sidic-glucose.	Acid c.c. N/10 NaOH.
1A	0	14.41	0.095	0.085	0.010	0.338	0.151	—	85.1
1B	0	14.51	0.142	0.081	0.061	0.350	0.140	—	98.0
Mean		14.46	0.119	0.083	0.036	0.344	0.146	—	91.6
2A	16	13.19	0.210	0.117	0.093	0.362	1.794	—	16.2
2B	16	12.76	0.192	0.083	0.109	0.382	1.968	—	15.2
E. 1A	16	11.52	0.158	0.084	0.074	0.066	1.590	—	54.1
E. 1B	16	12.03	0.140	0.082	0.058	0.036	1.422	—	31.9
Mean		12.37	0.175	0.092	0.084	0.212	1.694	—	29.4
3A	37	23.15	0.095	0.074	0.021	0.253	11.130	0.004	17.0
3B	37	23.10	0.094	0.067	0.028	0.277	11.210	0.004	17.6
E. 2A	37	27.20	0.137	0.090	0.047	0.256	16.48	0.027	15.2
E. 2B	37	21.96	0.138	0.095	0.043	0.033	13.42	0.019	6.9
Mean		23.85	0.116	0.082	0.035	0.205	13.06	0.014	14.2
4A	58	28.32	0.099	0.050	0.048	0.080	16.30	0.017	17.3
4B	58	29.00	0.094	0.052	0.041	0.124	15.61	0.034	16.2
E. 3A	58	27.47	0.102	0.053	0.049	0.136	17.18	0.030	15.3
E. 3B	58	28.06	0.095	0.055	0.040	0.181	19.10	0.030	13.9
Mean		28.21	0.098	0.053	0.045	0.130	17.05	0.028	15.7
5A	'80'	26.97	0.071	0.034	0.037	0.139	16.18	0.043	14.8
5B	'80'	27.08	0.075	0.024	0.051	0.095	16.28	0.043	11.5
E. 4A	79	26.85	0.086	0.055	0.031	0.050	17.89	0.016	9.2
E. 4B	79	26.53	0.066	0.036	0.030	0.057	16.47	0.024	10.8
Mean		26.86	0.075	0.037	0.037	0.085	16.71	0.032	11.6

## APPENDIX TABLE II

*Development on the Plant: Series (b). Composition of Pulp expressed as Percentages of Fresh Weight*

No.	Sampling date.	Approx. days from emergence.	Total dry matter.	Total reducing sugars.	Glucose.	Fructose.	Sucrose.	Starch.	Glyco-sides.	Acid c.c. N/10 NaOH.
A1	June 18	80	23.68	0.094	0.054	0.040	0.035	14.96	0.023	8.8
B1	"	80	27.70	0.098	0.059	0.039	0.074	18.28	0.024	8.6
Mean			25.69	0.096	0.057	0.040	0.055	16.62	0.024	8.7
A2	June 30	92	26.23	0.144	0.082	0.062	0.021	17.55	0.027	3.7
B2	"	92	26.44	0.151	0.095	0.056	0.007	16.04	0.040	8.8
Mean			26.34	0.148	0.089	0.059	0.014	16.80	0.034	6.2
A3	July 7	99	26.31	0.055	0.037	0.018	0.003	21.42	0.040	6.4
B3	"	99	25.76	0.052	0.038	0.014	0.017	21.98	0.032	5.6
Mean			26.04	0.054	0.038	0.016	0.010	21.70	0.036	6.0
A4	July 14	106	25.55	0.033	0.026	0.007	0.000	22.39	0.021	6.3
B4	"	106	25.57	0.060	0.028	0.032	0.023	20.04	0.054	8.3
Mean			25.56	0.047	0.027	0.020	0.012	21.22	0.038	7.3

APPENDIX TABLE II (cont.)—

No.	Sampling date.	Approx. days from emergence.	Total dry matter.	Total reducing sugars.	Glucose.	Fructose.	Sucrose.	Starch.	Glycosides.	Acid c.c. N/10 NaCh.
A5	July 21	111	25.28	0.271	0.156	0.115	0.000	16.81	0.151	11.6
B5	"	111	24.00	0.620	0.375	0.245	0.000	15.12	0.230	13.4
Mean			24.64	0.446	0.266	0.180	0.000	15.97	0.191	12.5
A6	July 24	114	25.03	0.069	0.056	0.013	0.046	15.68	0.043	7.8
B6	"	114	22.64	1.860	0.960	0.896	0.013	11.62	0.543	18.8
Mean			23.84	0.965	0.570	0.455	0.030	13.65	0.293	13.3
A7	July 28	118	24.56	0.149	0.112	0.037	0.000	14.06	0.070	8.0
B7 (i)	"	118	24.26	0.110	0.083	0.027	0.000	13.74	0.056	7.8
Mean			24.41	0.130	0.098	0.037	0.000	13.90	0.063	7.9
*B7 (ii)	"	118	20.01	7.07	3.897	3.172	0.000	3.472	2.562	38.1
A8	July 31	121	24.32	0.070	0.054	0.016	0.013	13.54	0.048	9.0
B8	"	121	22.75	0.148	0.095	0.053	0.000	12.15	0.029	11.1
Mean			23.54	0.109	0.075	0.035	0.007	12.85	0.039	10.1
A9	August 4	125	22.42	1.743	0.939	0.804	0.000	10.08	0.691	17.7
B9	"	125	22.95	0.677	0.441	0.236	0.028	11.74	0.339	16.5
Mean			22.69	1.210	0.690	0.520	0.014	10.91	0.515	17.1
A10	August 9	130	22.49	3.699	2.095	1.604	0.361	6.79	1.162	30.1

\* Yellowing fruit.

APPENDIX TABLE III

*Development on the Plant: Series (a). Composition of Skin expressed as Percentages of Fresh Weight*

No.	Days from emergence.	Total dry matter.	Total reducing sugars.	Glucose.	Fructose.	Sucrose.	Starch.	Glycosidic-glucose.	Acid c.c. N/10 NaOH.
1A	0	5.68	0.104	0.104	0.000	0.093	0.292	—	—
1B	0	6.10	0.268	0.102	0.166	0.120	0.291	—	—
Mean		5.89	0.186	0.103	0.083	0.107	0.292	—	—
2A	16	8.72	0.226	0.152	0.074	0.136	0.948	0.011	—
2B	16	8.55	0.233	0.168	0.065	0.124	1.497	0.012	—
E. 1A	16	8.52	0.115	0.091	0.024	0.038	0.429	0.012	2.4
E. 1B	16	8.10	0.112	0.096	0.016	0.073	0.393	0.006	1.4
Mean		8.47	0.172	0.127	0.045	0.093	0.817	0.010	1.9
3A	37	10.31	0.195	0.125	0.070	0.113	0.934	0.025	—
3B	37	10.64	0.168	0.100	0.068	0.169	1.710	0.014	—
E. 2A	37	11.61	0.113	0.098	0.015	0.069	2.439	0.020	3.0
E. 2B	37	11.47	0.152	0.105	0.047	0.044	2.397	0.025	1.3
Mean		11.01	0.157	0.107	0.050	0.099	1.870	0.021	2.2
4A	58	10.33	0.146	0.100	0.046	0.075	2.207	0.022	—
4B	58	10.89	0.101	0.070	0.031	0.126	2.078	0.020	—
E. 3A	58	11.24	0.103	0.075	0.028	0.043	2.456	0.028	3.5
E. 3B	58	11.30	0.086	0.065	0.021	0.059	2.262	0.022	1.0
Mean		10.94	0.109	0.078	0.032	0.076	2.251	0.023	2.3
5A	'80'	11.02	0.104	0.073	0.031	0.042	2.582	0.020	3.1
5B	'80'	11.55	0.081	0.061	0.020	0.071	2.597	0.019	4.0
E. 4A	79	9.78	0.090	0.062	0.028	0.020	2.259	0.017	5.4
E. 4B	79	9.70	0.058	0.041	0.017	0.050	1.923	0.037	3.5
Mean		10.51	0.084	0.059	0.024	0.046	2.340	0.024	4.0

## APPENDIX TABLE IV

*Development on the Plant: Series (b). Composition of Skin expressed as Percentages of Fresh Weight*

No.	Sampling date.	Approx. days from emergence.	Total dry matter.	Total reducing sugars.	Glucose.	Fructose.	Sucrose.	Starch.	Glyco-sides.	Acid c.c. N/10 NaOH.
A1	June 18	80	10.64	0.061	0.039	0.022	0.058	2.469	0.025	2.7
B1	"	80	10.69	0.071	0.048	0.023	0.076	2.479	0.029	1.2
Mean			10.67	0.066	0.044	0.023	0.067	2.474	0.027	2.0
A2	June 30	92	10.11	0.105	0.066	0.039	0.024	2.204	0.038	—
B2	"	92	9.82	0.084	0.062	0.022	0.034	2.025	0.028	—
Mean			9.97	0.095	0.064	0.031	0.029	2.115	0.033	—
A3	July 7	99	9.78	0.077	0.049	0.028	0.027	3.262	0.041	9.6
B3	"	99	10.11	0.069	0.050	0.019	0.035	3.078	0.035	10.6
Mean			9.95	0.073	0.050	0.024	0.031	3.170	0.038	10.1
A4	July 14	106	9.61	0.038	0.029	0.009	0.013	2.840	0.028	7.7
B4	"	106	9.74	0.045	0.034	0.011	0.011	2.541	0.030	10.0
Mean			9.68	0.042	0.032	0.011	0.012	2.691	0.029	8.9
A5	July 21	111	9.88	0.091	0.063	0.028	0.050	2.622	0.030	4.9
B5	"	111	10.02	0.096	0.062	0.033	0.047	3.327	0.038	8.4
Mean			9.96	0.094	0.063	0.031	0.049	2.975	0.034	6.7
A6	July 24	114	10.63	0.065	0.046	0.019	0.005	2.938	0.030	8.3
B6	"	114	9.14	0.225	0.148	0.077	0.062	2.334	0.111	6.4
Mean			9.89	0.145	0.097	0.048	0.034	2.636	0.071	7.4
A7	July 28	118	10.01	0.064	0.042	0.022	0.010	3.479	0.020	8.2
B7 (i)	"	118	8.92	0.089	0.063	0.026	0.042	2.241	0.052	6.8
Mean			9.46	0.077	0.053	0.024	0.026	2.860	0.036	7.5
*B7 (ii)	"	118	8.19	0.783	0.447	0.336	0.476	0.662	0.300	22.0
A8	July 31	121	9.76	0.060	0.025	0.035	0.012	3.137	0.021	6.9
B8	"	121	9.69	0.078	0.049	0.029	0.013	3.084	0.028	7.3
Mean			9.73	0.069	0.037	0.032	0.013	3.111	0.025	7.1
A9	August 4	125	9.79	0.412	0.216	0.196	0.083	2.390	0.137	7.0
B9	"	125	11.73	0.046	0.041	0.005	0.020	3.430	0.036	7.1
Mean			10.76	0.229	0.129	0.100	0.052	2.910	0.087	7.1
A10	August 9	130	11.62	0.807	0.510	0.297	0.203	2.361	0.189	18.5

\* Yellowing Fruit.

## APPENDIX TABLE V

*Development on the Plant: Triple Averages of the Percentage Composition Data for the Pulp*

Days (corrected).	Total dry matter.	Glucose.	Fructose.	T.R.S.	Sucrose.	Total sugars.	Starch.	Glyco-sides.	Acid c.c. N/10 NaOH.
<i>Series (a)</i>									
0	14.46	0.083	0.036	0.119	0.344	0.463	0.146	—	91.6
18½	16.90	0.089	0.052	0.141	0.254	0.395	4.97	—	45.2
37	21.48	0.076	0.055	0.131	0.183	0.314	10.60	0.014	19.8
58½	26.31	0.058	0.039	0.097	0.141	0.238	15.61	0.028	13.8
80	26.86	0.038	0.037	0.075	0.086	0.161	16.71	0.032	11.6
<i>Series (b)</i>									
80	25.69	0.057	0.040	0.097	0.055	0.152	16.62	0.024	8.7
89½	26.02	0.061	0.038	0.099	0.026	0.125	18.71	0.031	7.0
99	25.98	0.051	0.032	0.083	0.012	0.095	19.91	0.036	6.5
105	25.41	0.110	0.072	0.182	0.007	0.189	19.63	0.088	8.6
110	24.68	0.268	0.218	0.486	0.014	0.500	16.95	0.174	11.1
114½	24.30	0.291	0.196	0.487	0.010	0.497	14.51	0.182	11.3
117½	23.93	0.228	0.174	0.402	0.010	0.412	13.47	0.132	10.4
121½	23.55	0.288	0.196	0.484	0.005	0.489	12.55	0.208	11.7
125½	22.91	0.953	0.720	1.673	0.125	1.798	10.18	0.639	19.1
130	22.49	2.095	1.604	3.699	0.361	4.064	6.79	1.162	30.1

## APPENDIX TABLE VI

*Development on the Plant: Triple Averages of the Percentage Composition  
Data for the Skins*

Days (corrected). Series (a)	Total dry matter.	Glucose.	Fructose.	T.R.S.	Sucrose.	Total sugars.	Starch.	Glyco- sides.	Acid c.c. N/10 NaOH.
0	5.89	0.103	0.083	0.186	0.107	0.293	0.292	—	—
18½	8.45	0.113	0.059	0.172	0.100	0.272	0.993	0.011	1.9
37	10.14	0.104	0.042	0.146	0.089	0.235	1.646	0.019	2.1
58½	10.82	0.082	0.036	0.118	0.074	0.192	2.154	0.023	2.8
80	10.52	0.060	0.025	0.085	0.046	0.131	2.341	0.024	4.0
Series (b)									
80	10.67	0.044	0.023	0.066	0.067	0.133	2.474	0.027	2.0
89½	10.20	0.053	0.026	0.079	0.042	0.121	2.586	0.033	6.1
99	9.87	0.049	0.022	0.071	0.024	0.095	2.659	0.033	9.5
105	9.86	0.048	0.022	0.070	0.031	0.101	2.945	0.034	8.6
110	9.84	0.064	0.030	0.094	0.032	0.126	2.767	0.045	7.7
114½	9.76	0.071	0.034	0.105	0.036	0.141	2.824	0.047	7.2
117½	9.69	0.062	0.035	0.097	0.023	0.120	2.869	0.044	7.3
121½	9.98	0.073	0.052	0.125	0.030	0.155	2.960	0.049	7.2
125½	10.70	0.225	0.143	0.368	0.089	0.457	2.794	0.100	10.9
130	11.62	0.510	0.297	0.807	0.203	1.010	2.361	0.189	18.5

## APPENDIX TABLE VII

*Development on the Plant: Triple Averages of the Mean Values (gm.) for  
Total Amounts per Finger of the Carbohydrates, &c., in the Pulp*

Days (corrected). Series (a)	Pulp weight.	Total dry matter.	Glucose.	Fructose.	Sucrose.	Total sugars.	Starch.	Glyco- sides.	Acid c.c. N/10 NaOH.
0	1.4	0.203	0.001	0.001	0.005	0.007	0.001	—	1.29
18½	10.3	2.09	0.009	0.006	0.023	0.038	0.971	—	2.17
37	25.0	5.64	0.017	0.012	0.040	0.069	3.644	0.003	4.11
58½	43.9	11.82	0.022	0.018	0.054	0.094	7.12	0.013	6.30
80	64.9	17.54	0.023	0.026	0.060	0.109	10.76	0.023	8.75
Series (b)									
80	74.4	19.15	0.042	0.030	0.041	0.113	12.40	0.018	6.5
89½	98.4	25.60	0.060	0.037	0.026	0.123	18.41	0.030	6.9
99	124.9	32.41	0.064	0.040	0.015	0.119	24.84	0.045	8.1
105	145.9	37.19	0.295	0.105	0.011	0.411	28.60	0.128	12.6
110	159.6	39.38	0.428	0.322	0.020	0.770	27.02	0.243	15.4
114½	164.2	39.90	0.478	0.322	0.016	0.816	23.84	0.299	18.5
117½	171.4	41.01	0.391	0.298	0.017	0.706	23.04	0.226	17.9
121½	178.6	42.10	0.513	0.350	0.009	0.872	22.40	0.371	20.9
125½	162.4	37.21	1.550	1.169	0.203	2.922	16.52	1.039	31.0
130	119.1	26.80	2.501	1.911	0.430	4.842	8.09	1.384	35.8



## APPENDIX TABLE VIII

*Development on the Plant: Triple Averages of the Mean Values (gm.) for Total Amounts per Finger of the Carbohydrates, &c., in the Skins*

Days (corrected).	Skin weight.	Total dry matter.	Glucose.	Fructose.	Sucrose.	Total sugars.	Starch.	Glyco- sides.	Acid c.c. N/10 NaOH
<i>Series (a)</i>									
0	7.6	0.466	0.008	0.007	0.008	0.023	0.022	—	—
18½	27.8	2.702	0.032	0.015	0.028	0.075	0.380	0.004	0.55
37	43.2	5.51	0.044	0.019	0.039	0.102	0.776	0.009	0.93
58½	50.8	6.51	0.041	0.018	0.038	0.097	1.104	0.012	1.33
80	52.1	5.55	0.032	0.015	0.025	0.070	1.243	0.012	1.75
<i>Series (b)</i>									
80	61.7	6.57	0.027	0.014	0.041	0.082	1.522	0.017	1.2
89½	67.6	6.87	0.036	0.017	0.028	0.081	1.783	0.023	5.6
99	76.9	7.57	0.036	0.016	0.018	0.070	2.078	0.026	—
105	85.7	8.44	0.041	0.018	0.026	0.085	2.519	0.029	7.3
110	90.3	8.95	0.059	0.043	0.029	0.131	2.495	0.041	6.9
114½	91.0	8.99	0.069	0.050	0.033	0.152	2.566	0.044	6.5
117½	91.6	8.98	0.061	0.051	0.023	0.135	2.620	0.041	6.7
121½	93.4	9.29	0.077	0.057	0.052	0.186	2.734	0.049	6.8
125½	82.4	8.67	0.158	0.105	0.084	0.347	2.324	0.075	8.1
130	58.9	6.85	0.301	0.175	0.120	0.596	1.394	0.111	10.9

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## Studies in the Proteaceae

### IV. Structure and Development of the Ovule of *Hakea saligna* Knight

BY

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With sixteen Figures in the Text

#### INTRODUCTION

*HAKEA SALIGNA* Knight, a native of Queensland, Australia, is a medium-sized shrub with dense foliage; the plants make fine hedges, which are especially ornamental when there is a flush of copper-coloured young leaves. The inflorescences are slender and conform to a general racemose plan. The individual flowers are found on the main axis of the inflorescence in small clusters of 4 to 8. The flowers are distinctly zygomorphic and show two well-marked portions: a proximal tubular portion, and a distal pouch formed by a hook-like curvature of the united perianth lobes (Fig. 1). Inside the distal pouch the sessile stamens are found enclosing a large stigma which is discoid. The stigma contains a large funnel-like cavity forming an effective pollen-collecting apparatus. The tubular portion of the flower contains the long and slender style with the ovary situated at the base. The latter is raised on a short stalk, in front and on either side of which are two nectar-secreting scales (Figs. 1 and 2).

The material for the present study was obtained from plants grown at the gardens in Ootacamund, Mount Nilgiris, South India. Bouin's fluid was used in killing the material, and after the usual processes of dehydration and infiltration, sections were cut from 6 to 8 $\mu$  in thickness and stained in Heidenhain's iron-alum haematoxylin. The sectioning of the material was found to be very difficult as the ovary wall and the ovules contain plenty of tannin even in early stages. The difficulty could not be overcome by any means, and only after repeated attempts a few fairly satisfactory preparations were obtained.

#### THE OVULE

The ovary is made up of a single carpel and contains two amphitropous ovules attached laterally on the ventral side, and with the micropyles pointing downwards (Fig. 2). Very early in development the base of the ovule grows into a conical mass, which becomes very conspicuous later as the ovule develops further (Figs. 3 and 5). This conical mass contains a few tannin-cells, which steadily increase in number as the development of the ovule

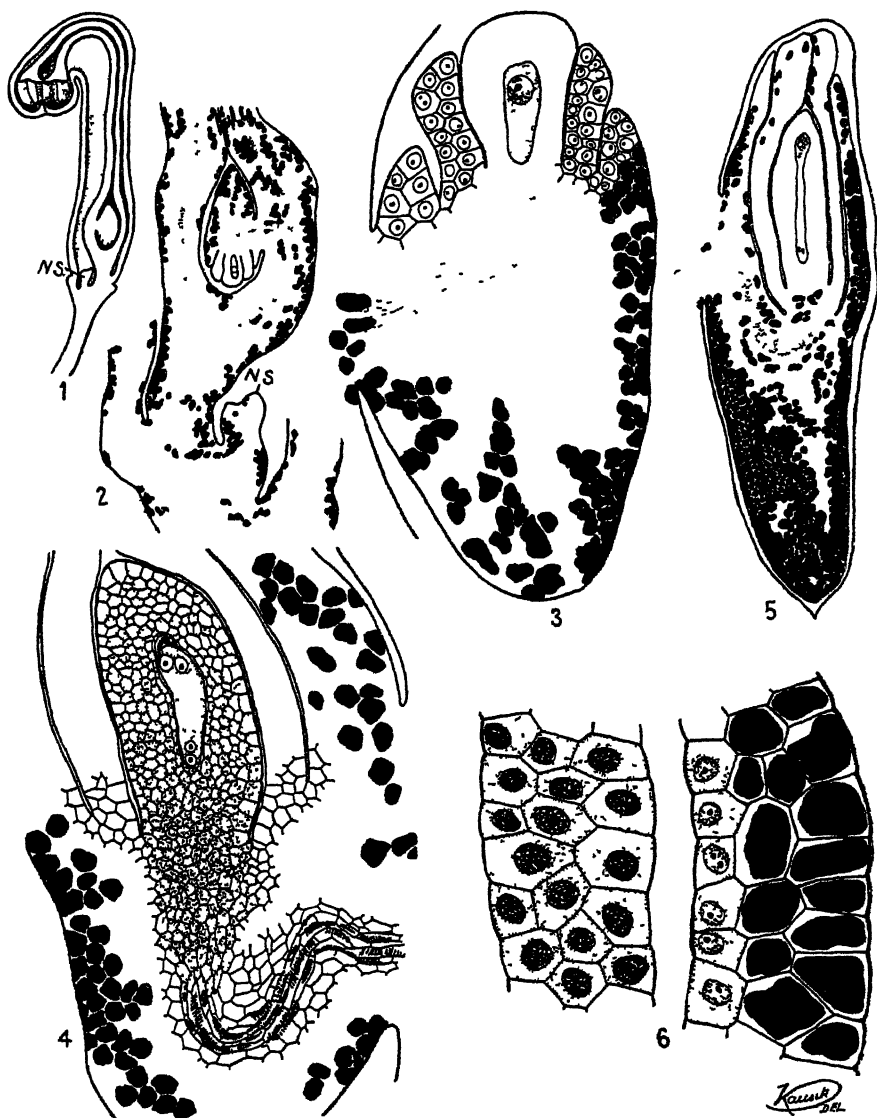
proceeds. Finally, the whole conical mass, which in the meanwhile becomes very enormous and characteristically formed, occupying almost half the entire ovule, contains abundant tannin in all its cells (Fig. 5). It is this extremely exaggerated base of the ovule with its tannin-containing cells, as also the ovary wall with its numerous similar cells, that makes the material quite hard for preparing satisfactorily serial microtome sections. Even in spite of great care and reducing the thickness of the sections to the lowest possible limit, the hard base of the ovule becomes, very frequently, scraped into quite a thick slice tearing away the inner softer tissues.

The ovule has two integuments, both of which have two layers of cells in the earlier stages (Fig. 3). The outer integument contains tannin in some of the cells of the outer layer, particularly towards the base of the ovule. Both the integuments later become three-layered generally (Fig. 6), but there may be four layers of cells at the base of the ovule, as the ovule grows further. All the cells of the inner integument now contain very darkly stained nuclei with a little cytoplasm; on the other hand, the cells of the innermost layer alone of the outer integument show clearly the nuclei, rather feebly stained, while all the cells of the outer two layers contain tannin. The growth of the integuments is at first slow, but after a time the inner integument grows more rapidly and completely invests the nucellus to form a long micropyle (Fig. 5). The outer integument, on the contrary, lags behind and reaches only the lower portion of the micropyle.

The nucellus, even in a young ovule, consists of a large mass of cells (Fig. 7). During the further growth of the ovule and the development of the embryo-sac, the nucellus becomes more and more massive. It consists of from four to six layers of cells all along the sides of the embryo-sac, while it is more extensive below (Fig. 4). The larger amount of nucellus at the base does not, however, appear to be due to the presence of any meristematic region as is characteristically seen in the chalaza, and becomes active, especially after fertilization, in the developing ovule of *Grevillea* (Brough, 1933; Kausik, 1938a). A definite chalazal meristematic zone with regularly dividing cells forming additional nutritive layers could not, for this reason, be identified at any stage in the development of the ovule of *Hakea saligna*. The distal region of the nucellus formed as a parietal tissue overlying the developing embryo-sac is not quite glandular in appearance, nor do the outer epidermal cells of this region form prominent glandular papillae (Fig. 4) as in the other members of the family studied previously. In fact, the cells of the nucellus at the distal region are not as rich in nutritive contents as those more proximally situated.

The vascular strand of the ovule, which is connected on to the main vascular strand traversing the ovary wall (Fig. 2), is arched over towards the base of the nucellus (Fig. 3). As the ovule becomes older, the vascular strand undergoes a more complete curvature with a hairpin-like bend towards the base of the ovule (Fig. 5).

The nucellus contains a single archesporial cell which gives rise to the



FIGS. 1-6. Fig. 1. Longitudinal section of young flower in sagittal plane showing zygomorphy and the distinction into the distal pouch and the proximal tubular portion.  $\times 6$ . Fig. 2. Longitudinal section of ovary showing the amphitropous ovule.  $\times 80$ . Fig. 3. Section of a young ovule containing the megaspore mother cell; the two layers of cells in the integuments are shown.  $\times 400$ . Fig. 4. Section of an older ovule showing the large nucellus with a four-nucleate embryo-sac; only portions of the integuments are shown here. The distribution of nutrition in the different regions of the nucellus is indicated by differential stippling.  $\times 600$ . Fig. 5. Section of ovule containing a fully developed embryo-sac which is extremely long and narrow. The conical projection formed by the base of the ovule is very conspicuous here.  $\times 120$ . Fig. 6. Portions of the integuments from the ovule in Fig. 5 enlarged to show the details.  $\times 1,350$ . N.S. nectar-secreting scale; tannin is represented by black areas.

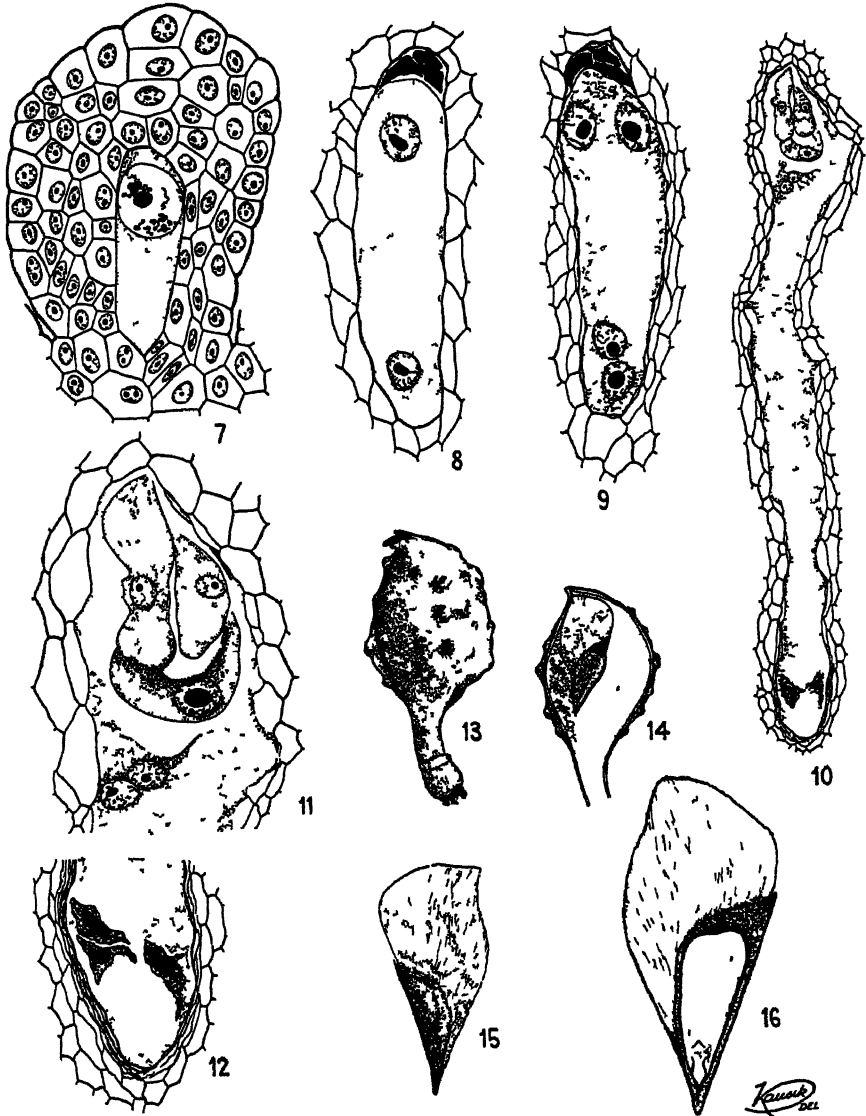
megaspore mother cell and a large parietal tissue. The megaspore mother cell enlarges rapidly (Fig. 7). The divisions of this cell could not be followed, but it may be assumed that the course of development is normal as in the other members of the family, and that the lowermost megaspore develops into the typical eight-nucleate embryo-sac. The disorganized remnants of the upper three megaspores are clearly seen at the two- and four-nucleate stages of the embryo-sac (Figs. 8 and 9). During the development of the embryo-sac the adjacent one or two layers of the nucellus become crushed. The fully developed embryo-sac is extremely elongated and narrow (Fig. 10). Its outline is irregular in places on account of the slight encroachment made by it on the surrounding nucellus. The embryo-sac contains, at the micropylar end, two synergids showing a slightly differentiated filiform apparatus, a large egg-nucleus lying in a dense mass of cytoplasm, and two polar nuclei (Fig. 11). The antipodal end is very deeply situated (Fig. 10), reaching almost to the base of the nucellus where the vascular strand of the ovule terminates. It contains the three antipodal cells which are very darkly staining and seem to disorganize very early (Fig. 12). The centre of the embryo-sac contains a much vacuolated cytoplasm, which partly results from the dissolution of the adjoining cells of the nucellus.

During the formation of the mature embryo-sac the cells along the median region of the nucellus are destroyed. On account of the absence of a definite meristematic zone in the chalaza, additional layers of nucellus with a nutritive function are not intercalated in the ovule; the embryo-sac has, therefore, to increase in size, in subsequent stages after fertilization, only along the sides where the cells of the nucellus are still intact. This presents a strong contrast to *Grevillea*, where fresh cells are added at the base of the nucellus by the activity of a chalazal meristematic zone and these cells are used up after fertilization by the haustorial *vermiform appendage* of the endosperm (Kausik, 1938 *a* and 1939).

Suitable preparations of the material showing either fertilization or early stages in the development of the embryo and the endosperm were not available. This is due to the difficulty of cutting the material at such stages on account of the general hardness which becomes progressive as the ovule develops.

The oldest embryo observed is very large and occupies completely the cavity inside the seed (Fig. 16) formed as a result of a total destruction of the nucellus. The embryo has two large cotyledons, each with two conspicuous basal lobes enclosing the proximal portion of the radicle. The distal portion of the radicle has a pointed root-cap fitting into the long micropyle. The hypocotyl is short and stout with a slightly conical apex.

In the development of the seed, the inner integument forms a thin but tough and hard protective covering for the embryo. The outer integument becomes flattened along the antero-posterior plane of the seed and develops obliquely more at the base of the seed to form a membranous wing (Figs. 15 and 16).



FIGS 7-16 Fig 7. Megaspore mother cell and the overlying parietal tissue  $\times 900$ . Fig. 8 A two-nucleate embryo-sac  $\times 1,350$  Fig 9 A four-nucleate embryo-sac  $\times 900$ . In Figs 8 and 9 the disorganized upper three megaspores are seen Fig 10. A fully developed embryo-sac from the ovule in Fig 5  $\times 600$  Figs 11 and 12. The micropylar and the antipodal ends respectively of the embryo-sac enlarged from Fig 10  $\times 1,800$ . Fig. 13. External aspect of the fruit showing the tubercles and the two beak-like projections at the apex, lying on either side and in front of the persisting base of the style.  $\times 1$ . Fig. 14. Longitudinal section of fruit to show the thick endocarp and the seed *in situ*  $\times 1$ . Fig. 15. Seed showing the membranous wing.  $\times 15$ . Fig 16. Longitudinal section of seed showing the large embryo.  $\times 25$ .



The wing imparts a characteristic gyrating movement to the seed during its dissemination.

The fruit develops a hard and woody endocarp, which is very thick (Fig. 14). The outer surface of the fruit presents a strongly tuberculate appearance. The apex has two curious beak-like projections, between and slightly behind which is the persisting base of the style in the form of a hard spine (Fig. 13). The presence of the two apical beak-like projections is, perhaps, of significance when a comparison is made between these and similarly formed elevations described in *Macadamia ternifolia* (Kausik, 1938*b*), where they are regarded, following the interpretation of the ovary by Hunt (1937), as perhaps representing the outer sterile lobes of a primitive carpel.

While generally both the seeds of a fruit attain equal development, occasionally one of the seeds is crushed with its embryo shrivelled up, while the other alone grows fully and contains a perfectly developed embryo.

#### CONCLUSIONS

While the general hardness of the floral parts and abundance of tannin-cells are characters common to all the members of the Proteaceae so far studied, these are even more pronounced in *Hakea saligna*. The plants of the family are generally very difficult to be handled with ease while preparing microtome sections. It is perhaps this difficulty in obtaining suitable preparations of the different stages of development that is largely responsible for the very limited number of investigations in the literature of the family. Our knowledge of floral development in the several genera is, therefore, necessarily meagre. The family, otherwise, has many unusual and varied features which may be disclosed only after persistent and careful study. It is of interest to note here that Brough (1933) regarded *Grevillea robusta* as the most favourable of the several genera after a very judicious selection. A much earlier worker, Ballantine (1909), experienced considerable difficulties in the case of *Protea Lepidocarpon*, in which fertilization and early stages of embryo and endosperm could never be followed.

Although in general the development of the ovule and the embryo-sac conforms to that in the other members of the family already studied, there are notable departures. The inner integument, growing at first only slowly, very soon covers the entire nucellus and forms a long micropyle. In *Macadamia* (Kausik, 1938*b*) the apex of the nucellus is partly exposed when the embryo-sac is ready for fertilization. The apical region of the nucellus in *Hakea saligna* does not appear to be glandular. Further, the overlying epidermal cells do not grow out as conspicuously formed papillae, as in either *Grevillea* (Brough, 1933; Kausik, 1938*a* and 1939) or *Macadamia* (Kausik, 1938*b*). The base of the nucellus seems to lack a definite meristematic region; consequently, only a limited nutritive tissue is found at the chalaza. This feature is of significance when we consider further the fact that the embryo-sac grows largely only along the longitudinal direction before fertilization; its growth in subse-

quent stages, namely, after fertilization, is mainly restricted to the sides where the nucellus is still intact. It may, therefore, be reasonably inferred that the lack of additional continually forming nutritive cells at the chalaza is the cause of a suppressed activity of the lower end of the embryo-sac during post-fertilization stages. Consequently, a *vermiform appendage* as described in *Grevillea robusta* (Kausik, 1938a) and *G. Banksii* (Kausik, 1939) or any corresponding structure is, perhaps, absent in *Hakea saligna*. This point could not, however, be verified actually for want of suitable material at these stages.

A certain interest is attached to *Hakea* as indicating the existence of the Proteaceae in the earlier geological strata. Reid and Reid (1915)<sup>1</sup> have described a specimen, presumably the fruit of a *Hakea* from the Pliocene deposits in Europe. This is of value in suggesting the antiquity of the family and indicating its wider distribution in the past. The authors state: 'In the Pliocene deposits of Brunssum has been found the half of a hard woody fruit, so like that of *Hakea*, and so unlike anything else that we can find, that we feel compelled to treat it as an unrecorded species of this genus, and perhaps therefore the last representative of this dying out family in Europe.' In this connexion Seward (1931) remarks that 'A few fossils from Pliocene beds in Europe have been assigned to the Proteaceae and though the identifications are regarded by some authors with suspicion, their validity has not been disproved.'

#### SUMMARY

The base of the ovule of *Hakea saligna* Knight grows enormously and forms a hard conical projection all the cells of which contain tannin.

The zone of meristematic cells which is characteristic of the chalazal region of the ovule of *Grevillea* appears to be absent here.

The development of the embryo-sac is normal. When fully formed it is extremely long and narrow; the antipodal end of the sac reaches almost the base of the nucellus, even at the time of fertilization.

As a consequence of the absence of a chalazal meristematic zone the base of the nucellus does not appear to have additional nutritive cells. This perhaps accounts to some extent for the suppression of the activity of the antipodal end of the embryo-sac. The *vermiform appendage* of the endosperm, found in the post-fertilization stages of the ovule of *Grevillea*, is absent in *Hakea saligna*.

Reference is made in the paper to the existence of *Hakea* in the Pliocene beds of Europe. This is important in estimating the antiquity of the family as a whole and also its wider distribution in the past.

In conclusion the writer has pleasure in recording his appreciation and gratitude to Prof. M. A. Sampathkumaran, University of Mysore, for his

<sup>1</sup> The writer is grateful to Prof. B. Sahni, F.R.S., of Lucknow, India, for this reference.

continued interest and encouragement in these studies. Further, the writer's thanks are due to Mr. K. Subrahmanyam, of the staff of the Fernhill Palace of the Maharaja of Mysore, Ootacamund, Mount Nilgiris, South India, for kindly supplying the material for this investigation.

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# Morphological and Cytological Studies in the Genus *Calceolaria*<sup>1</sup>

## III. Meiosis in a Triploid *Calceolaria*

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With sixty Figures in the Text

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## I. INTRODUCTION

THE character of a triploid depends on the presence of three sets of chromosomes, which may either be identical (autotriploid) or dissimilar in their constitution (allotriploid). The phenomenon of triploidy was discovered by Gates (1909) when he noticed a plant with  $2n = 21$  chromosomes in hybrid cultures of a cross between *Oenothera lata* and *Oe. Lamarckiana*. He inferred that this was a result of hybridization between *Oe. lata* and *Oe. gigas*. A large number of triploids have been described since then, having arisen in different ways. Amongst well-known autotriploids, some of which are vegetatively propagated, may be mentioned: *Canna* (Belling, 1921, 1925), *Morus* (Osawa, 1920), *Hemerocallis* (Belling, 1925; Dark, 1932), *Tulipa* (Newton and Darlington, 1929), *Hyacinthus* (Belling, 1929), *Crocus* (Karasawa, 1933), *Thea* (Karasawa, 1932), *Narcissus* (Nagao, 1929), *Fritillaria* (Darlington, 1936), *Nasturtium* (Manton, 1935). Allotriploids have arisen in crosses between diploids and tetraploids and also in interspecific and intergeneric crosses. Species hybrids of *Triticum* (Thompson, 1926; Mather, 1935), *Avena* (Nishiyama, 1929), *Gossypium* (Skovsted, 1934) are examples. As intergeneric hybrids we may mention those of *Zea-Euchlaena* (Longley, 1934), *Raphano-Brassica* (Karpechenko, 1928), and *Citrus* × *Fortunella* (Longley, 1924). Triploids have turned up in experiment as mutants and

<sup>1</sup> Part III of thesis accepted for the Ph.D. degree of the University of London.

[Annals of Botany, N.S. Vol. IV, No. 13, January 1940.]

hybrids as in *Zea* (McClintock, 1929), *Solanum* (Lesley, 1928), *Primula* (Dark, 1931), *Nicotiana* (East, 1933), and *Oryza* (Nakamori, 1932; Morinaga and Fukushima, 1935).

The sudden appearance of triploids among diploids is usually believed to be the product of a haploid gamete with an unreduced diploid gamete or the result of fertilization of a haploid egg by two male nuclei, i.e. dispermy. This was first suggested by Gates (1924) as the cause of spontaneously occurring triploid mutants. When triploidy was independently observed in *Oenothera* by Lutz and Stomps (1912), they simply assumed that such triploid forms must have arisen through the union of a diploid with a haploid gamete. Gates (1915) first reported a process which might lead to the formation of a pollen grain with double the normal number of chromosomes. The only direct observation of the origin of triploidy is that of Ishikawa (1918), who found an embryo-sac in which two male nuclei were fusing with the egg nucleus, while a third had united with the polar nucleus. Neměc (1912) also observed two male nuclei uniting with the egg in *Gagea lutea*. Rhodes (1936) was able to raise a triploid in *Zea Mays*, with the male parent having the diploid complement of chromosomes and fusing with a normal egg. A similar explanation has been offered by Morinaga and Fukushima (1935) for the origin of a number of rice triploids in their cultures. Newman (1934) in *Acacia* was able to trace sometimes two pollen tubes in the nucellus going to the embryo-sac at the time of fertilization. Geerts (1909) found a tetraploid megaspore mother-cell which would lead to the formation of a diploid egg.

The appearance of gametes with the somatic number has been known in several cases. Hybrids, which are generally characterized by irregular meiosis, produce as a consequence a certain number of diploid and other non-typical gametes. Under the stimulation of extremes of temperature or other abnormal physiological conditions, many plants have been induced to produce diploid or polyploid gametes. By subjecting buds from the parent plant, as well as seeds and seedlings, to X-rays, Goodspeed (1930) produced both triploid and tetraploid plants in *Nicotiana Tabacum*. Triploids have also been obtained by treatment with colchicine. The origin of autotriploids, as in *Solanum melongena* (Janakiammal, 1934), *Solanum Lycopersicum* (Lesley, 1928), and *Zea Mays* (McClintock, 1929), has been inferred to be the result of fusion of reduced and unreduced gametes.

Syndiploidy (Strasburger, 1907) is a well-known phenomenon and may lead to doubling of chromosomes in gametes. It has been noticed in many plants: *Lactuca* (Gates and Rees, 1921), *Zea* (Randolph and McClintock, 1926), *Triticum compactum* (Gaines and Aase, 1926), *Nicotiana* (Ruttle, 1928), *Chrysanthemum* (Shimotamai, 1931), *Avena*, (Nishiyama, 1931), *Brassica japonica* (Fukushima, 1931), rice (Nandi, 1936). Nandi explained this as due to the failure of reduction in a pollen mother-cell or a fusion of pollen mother-cells prior to reduction. The important consequence of syndiploidy is the formation of unreduced gametes.

While accepting the general principle that the production of triploid hybrids is dependent on the fusion of an unreduced gamete and a reduced one, that diploid eggs are more functional than diploid sperms in the production of triploids is a widely held view (Watkins, 1932; Steere, 1932). Watkins thinks that there is a definite relationship between the relative proportion of chromosome numbers in the pollen tube and the style. He says that in cases where the chromosome numbers are in the ratio 1 : less than 2, the growth of the pollen tube is retarded. From this conclusion it follows that diploid pollen is not likely to function in the diploid to produce triploids. It is well known that most triploids are obtained as crosses between tetraploids as female parents and diploids as male. As instances we may cite the fact that diploid forms of *Primula sinensis*, *Datura Stramonium*, *Solanum Lycopersicum*, *Campanula persicifolia* when used as females in crosses with tetraploid forms set no seed. Steere made reciprocal crosses between *Petunia* tetraploids and diploids. In the cross  $2n \times 4n$ , the few plants obtained were diploids. But the reciprocal cross gave rise to triploids. He thinks that while a diploid sperm nucleus may rarely function, the diploid chromosome set of a triploid is almost invariably obtained from the egg. Instances are not wanting where triploids have been successfully obtained by crossing a tetraploid ♂ with a diploid ♀, e.g. *Nicotiana glutinosa* ( $2n = 24$ )  $\times$  *N. Tabacum purpurea* ( $2n = 48$ ); *Rubus rusticanus inermis* ( $2n = 14$ )  $\times$  *R. thyrsifer* ( $2n = 28$ ); *Phleum pratense* ( $2n = 14$ )  $\times$  *P. alpinum* ( $2n = 28$ ). Dermen (1931) secured triploids by the  $2n \times 4n$  cross in *Petunia*.

The somatic origin of a triploid is very rare. Perhaps the only case known so far is that reported by Huskins (1934) in *Lycopersicum* where the triploid arose somatically from a diploid stock. Ramanujam (1937) suggests that a triploid can arise without a sexual fusion, by somatic reduction in a hexaploid cell.

## II. MATERIAL AND METHODS

Material for this investigation was collected at the University Botanical Gardens, Cambridge, and the John Innes Horticultural Institution. I am thankful to the Directors of these two institutions for their kindness in allowing me to collect flower buds. The hybrid 62/30 was originally raised by the late Dr. E. J. Collins, of the John Innes Institution. It arose as a cross between *C. purpurea* and a hybrid variety.

No small difficulty was encountered in securing a proper fixation of the pollen mother-cell, on account of the heavy coating of resin on the flower buds and the resistant nature of the wall of the anther. The material was fixed at 6 p.m. when the divisions were at the peak. Active division was taking place also at about 11 a.m. But it is probable that the time of division changes with the time of the year, even when the material is grown in a hothouse. The calyx and corolla were always clipped off, the buds dipped in Carnoy's fluid for about 30 seconds, and then immersed in the fixative. Belling's modification

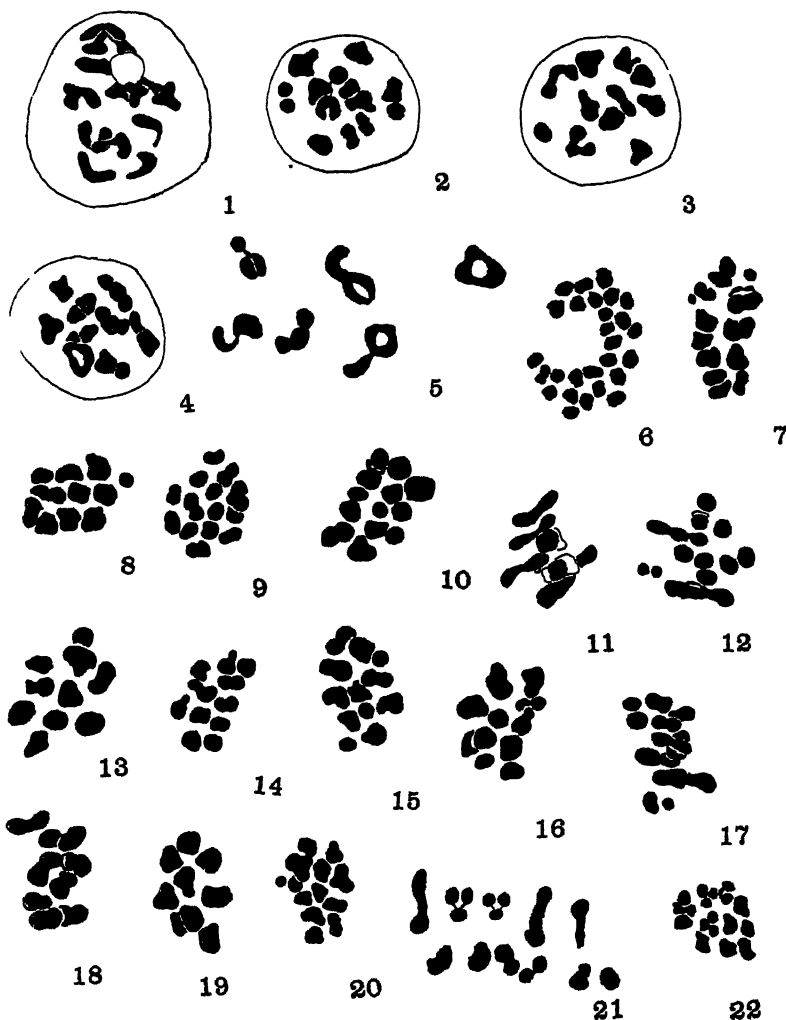
of Navashin was almost exclusively used as this gave uniformly good results. The fixed material was thoroughly washed in tepid water, dehydrated and embedded in hard wax (m.p.  $52^{\circ}$ ). The buds were sectioned at  $14\mu$  and stained with Newton's gentian violet schedule without a mordant. Most of the observations recorded here were made on smeared material. Anthers known to contain dividing pollen mother-cells were dissected out and laid on a clean slide. One tip of each anther was removed with a sharp knife and the anther gently pressed by means of another slide. The pollen mother-cells adhered to the slides, which were then inverted into the fixative. Belling's Navashin was used for the smears also. Newton's gentian violet-iodine technique was used along with chromic acid as a mordant. The mordant definitely improves the quality of staining of the chromatin.

No previous cytological work has been done on the genus *Calceolaria*.

At metaphase of somatic mitosis in the root-tips the triploid has twenty-seven chromosomes (Fig. 60). Variations in chromosome length make it difficult to pick out the diploid set. There are three satellited chromosomes; two of these have median constrictions, while the third has a clear submedian constriction. The heteromorphic nature of the satellited chromosomes is in itself a clear indication of the hybrid nature of the triploid. The presence of three satellited chromosomes is in accordance with de Mol's observation (1927) in *Hyacinthus*, that the number of nucleoli formed at telophase increased with polyploidy, diploids having two nucleoli, triploids three, tetraploids four and so on.

### III. DESCRIPTION OF MEIOSIS

*Meiosis.* The chromosomes of *Calceolaria* are so small that critical study of the prophases was not possible. At diakinesis three chromosomes were found attached to the nucleolus (Fig. 1). They would correspond to the three satellited chromosomes found in the mitotic metaphase. In a few cells the chromosomes at diakinesis were arranged in nine groups of three each (Fig. 4). But in the majority of cells the chromosomes appeared as trivalents, bivalents, and univalents in varying proportions. The most frequent configuration was  $5_{III} + 6_{II}$  (Fig. 3). The lowest degree of pairing observed in a cell was  $1_{III} + 11_{II} + 2_1$  and the highest was  $9_{III}$ . In one pollen mother-cell at metaphase I all the chromosomes appeared as univalents, pairing having completely failed (Fig. 6). Out of more than 400 cells studied, only a single instance of this condition was found. The organization of the total chromosome complement into trivalents in an allotriploid is very rare and has so far been reported in very few cases. In the majority of triploids studied, only a partial production of trivalent chromosomes has been reported. Chromosome pairing in triploids of the AAB and the ABC type has been studied in detail in several triploids. Steere (1932) finds in triploid hybrids of *Petunia* the chromosomes regularly forming trivalents at diakinesis. Belling (1928) observed in triploid *Hyacinths* that the twenty-four chromosomes sometimes



FIGS. 1-22. All figures were drawn with a camera lucida at bench level. An achromatic objective N.A.1.3 was used in conjunction with an  $\times 30$  ocular, giving an initial magnification of 5,400 diameters. The figures have been reduced to a half in reproduction. Fig. 1. p.m. cell in diakinesis with three univalents attached to the nucleolus. Figs. 2, 3. p.m. cells in diakinesis showing  $5_{III}+6_{II}$ . Fig. 4. p.m. cell in diakinesis showing  $9_{III}$ . Fig. 5. Types of trivalents at diakinesis. Fig. 6. Polar view of metaphase I showing 27 univalents. Fig. 7. Side view of metaphase I.  $11_{II}+2_{I}+1_{III}$ . Fig. 8. Polar view of metaphase I.  $6_{III}+4_{II}+1_{I}$ . Fig. 9. Polar view of metaphase I.  $11_{II}+2_{I}+1_{III}$ . Fig. 10. Side view of metaphase I.  $7_{III}+3_{II}$ . Fig. 11. Side view of metaphase I.  $7_{III}+3_{II}$ . Fig. 12. Side view of metaphase I.  $11_{II}+1_{III}+2_{I}$ . Fig. 13. Side view of metaphase I.  $7_{III}+3_{II}$ . Fig. 14. Side view of metaphase I.  $6_{III}+4_{II}+1_{I}$ . Fig. 15. Side view of metaphase I.  $7_{III}+4_{II}+1_{I}$ . Fig. 16. Side view of metaphase I.  $6_{III}+4_{II}+1_{I}$ . Fig. 17. Side view of metaphase I.  $7_{III}+4_{II}+1_{I}$ . Fig. 18. Side view of metaphase I.  $9_{III}$ . Fig. 19. Polar view of metaphase I.  $9_{III}$ . Fig. 20. Side view of metaphase I.  $1_{I}+2_{III}+10_{II}$ . Fig. 21. Side view of metaphase I drawn separately.  $5_{III}+6_{II}$ . Fig. 22. Polar view of metaphase I.  $5_{III}+6_{II}$ .



appeared as eight trivalents. The somatic complement in this triploid was made up of six short, six medium, and twelve long chromosomes. When eight trivalents were found, there were four large, two medium, and two small. This configuration agrees perfectly with the size relations of the somatic complement. Other triploids in which trivalents are commonly formed to the basic number are *Canna*, *Datura*, and *Hyacinthus* (Belling, 1921, 1927, 1928). In the  $F_1$  of *Nicotiana glutinosa*  $\times$  *Tabacum*, Clausen and Goodspeed (1925) reported that the thirty-six chromosomes of *glutinosa*  $\times$  *Tabacum* conjugate 'loosely' according to the *Boreale* scheme of Täckholm (1922). This was confirmed by Müntzing (1935), who found that at metaphase I in this hybrid, most of the thirty-six chromosomes appeared as univalents, but as a rule a few bivalents were also present and even occasional trivalents were seen. In hybrids of *Salix* (Häkansson, 1929), where the third set is mixed, sixteen to nineteen trivalents were found. In hybrids of *Galeopsis* (Müntzing, 1930), *Raphano-Brassica* (Karpechenko, 1938) and *Pygaera* (Federley, 1931) no trivalents were formed, all the chromosomes appearing as bivalents and univalents. Amongst triploids of the AAB type, Steere's *Petunia* hybrids represent the only reported instance showing complete trivalency. Darlington (1928) reports that in a hybrid of *Prunus avium*  $\times$  *cerasus* (a hybrid triploid of the ABC type; cross between a diploid and an allotetraploid species) the proportion of trivalents varies from one to eight. Other interesting hybrids of this constitution are *Avena* ( $3b = 21$ ) with a maximum of five trivalents (Nishiyama, 1934); hybrids of *Triticum aegilopoides*  $\times$  *dicoccum* with only three trivalents.

The varying formation of trivalents in the triploids described above is explained on the basis of random formation of chiasmata at pachytene. In triploids of the AAB type, the two similar sets usually pair off to form bivalents and the third set is left behind, appearing as univalents, as shown in triploids of rice (Ramanujam, 1937), wheat (Thompson, 1926), oats (Nishiyama, 1929), and *Raphano-Brassica* (Karpechenko, 1928).

The proportion of trivalents in the triploid *Calceolaria* studied is very variable, but its frequency is very high for an allotriploid. Usually a number of unpaired chromosomes are found, showing in most cases no particular attraction to any bivalent. Interstitial chiasmata are still found at early diakinesis, while by the end of this stage complete terminalization has usually occurred. When chiasmata joining three or four chromosomes are terminalized to the same end, a multiple chiasma is formed. Each end of a chromosome in a triploid may be associated with all the ends that are homologous with it or with some of them. The chromosomes making up the trivalents are united in different ways, the most common type being one where the third member is attached at one point of union of a pair. Illustrations of the types of trivalents found are given in Fig. 5. I never found the triple arc which involves four chiasmata. The chain trivalent with completed terminalization involving two terminal chiasmata is not frequent. But the Y trivalent where

all three chromosomes meet in a 'triple chiasma' which is produced by terminalization in the same direction of two interstitial chiasmata between three chromosomes, is frequent. The frequent appearance of the trivalent where the third member is attached to the point of union of a pair indicates 'loose affinity'. Very often this member is found detached from the association at metaphase and lying close to it, a fact also recorded by Müntzing (1933) in his triploid *Solanum tuberosum*. The ring trivalent is not of frequent occurrence. One ring trivalent has been figured in Text-figs. 4, 5.

Belling and Blakeslee (1926) first suggested the hypothesis that chromosomes are associated only at their homologous ends. This has been supported by numerous workers and has been applied to the explanation of the association of chromosomes. But a ring of three or the triangle (Fig. 4) cannot be explained on the basis of this hypothesis when all three chromosomes had different attractions at both ends. Huskins (1928) in wheat, and Meurman (1929) in *Prunus* found similar figures in which, however, one of the chromosomes lay transversely and was connected at the same end to the two adjacent chromosomes. Darlington (1928), in *Prunus*, also observed the closed ring or the triangle configuration of three chromosomes, which he interpreted to have been associated by the mutual attraction of the chromosomes, since the juxtaposition of homologous ends of chromosomes to make the three sides of a triangle is impossible. Belling and Blakeslee (1926, 1927) reported the appearance of the ring-of-three association in secondary trisomic *Datura*, which they explained by the segmental interchange hypothesis. On the assumption that only homologous parts pair, the occurrence of a ring of three is explained if we further assume that one of the components of the ring trivalent has two homologous ends. Such a chromosome would have the constitution AA, for instance, if the other two members were AB and BA. In triploid *Petunia* hybrids, Matsuda (1935) found ring trivalents in which all the three chromosomes were connected end to end. He suggests that probably reversed crossing over between homologous chromosomes had taken place in an ancestor, so that one of the chromosomes attained the same attraction at both ends.

A definite and specific association of any three homologues, giving a constant morphologically recognizable trivalent, does not exist. Such an occurrence of specific types of trivalents forming very often would be in keeping with the general expectations from a hybrid triploid, in which it is natural to expect the various chromosomes to show different degrees of homology. Mather (1935), in his triploid wheat hybrid, found a constant formation of two chain trivalents, and he explains that they are formed by the same chromosomes whenever they appear, due to autosyndesis between the two sets of the tetraploid parent and one of the diploid.

At metaphase I most of the chromosomes are found in these trivalent associations. But the trivalent configuration in which the third member is 'loosely' attached to the point of union of a pair is often found disrupted.

Fig. 6 represents a polar view of metaphase I in which the twenty-seven chromosomes appear as univalents owing to a total failure of pairing. In Fig. 18 the other extreme, in which there is complete trivalency, is illustrated. Fig. 19 is the same condition but a nearly polar view. The lowest degree of pairing, as was pointed out earlier, was  $1_{III} + 2_I + 11_{II}$  (Figs. 7 and 9). Figs. 6 to 22 illustrate the different degrees of pairing observed at metaphase I. The most frequent configuration is given in Fig. 16 ( $6_{II} + 5_{III}$ ). Fig. 21 represents the same condition at metaphase I, in side view, drawn separately.

The maximum number of univalents observed at diakinesis to metaphase is five. This is a relatively low number in a triploid. They usually lie clear of the plate (Fig. 12) as is usual in hybrids. Thompson (1926) in his triploid wheat found all the univalents in the plate, and that after the bivalents had divided all the univalents arranged themselves at the equator in a regular fashion and divided equationally. Sax (1922) has recorded quite a different behaviour in the triploid wheats he studied. None of the univalents here moved into the equatorial position.

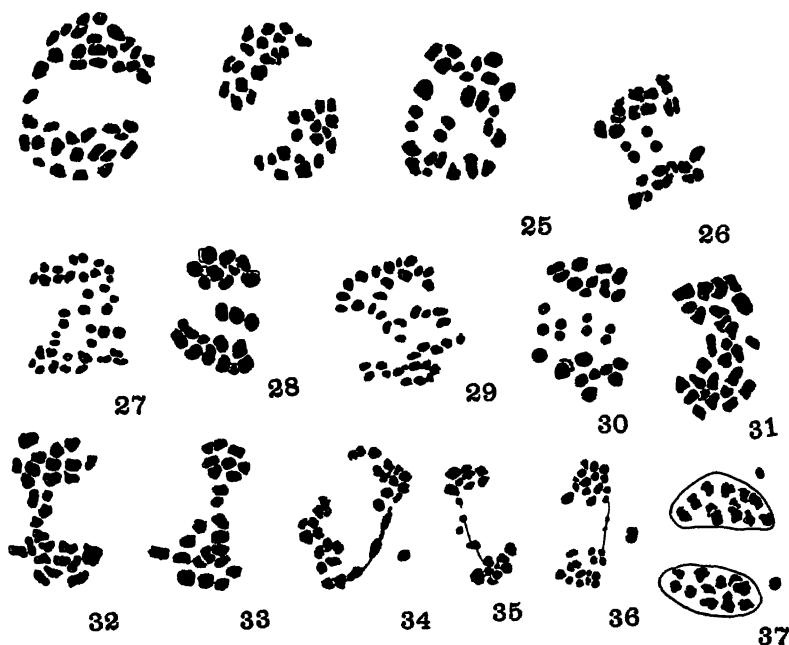
A haphazard arrangement of the chromosomes at metaphase I in triploids is a frequent occurrence (McClintock, 1929; Collins, 1933; Morinaga and Fukushima, 1935; Darlington, 1936; Karasawa, 1932). This irregularity is obviously due to the asymmetrical nature of the trivalents, in which the presence of three centromeres interferes with the normal orientation of compound bodies on the equator.

At anaphase I the distribution of the single and unpaired chromosomes is entirely at random. The simplest separation would be amongst those associations with all chiasmata terminal. The irregular orientation of the trivalents on the equator causes the anaphase to be very irregular.

The trivalents may break up into a bivalent and a univalent or three univalents. The members of the bivalents segregate regularly. In general, the univalents do not divide at first division but are assorted to the poles at random. This is supported by the fact that lagging chromosomes are very rare at the second division. Müntzing (1933) found a similar behaviour of the univalents in his triploid *Solanums*. There are several triploids in which the first division is clean while the divided univalents lag on the second division spindle (Lesley, 1926). Occasionally, however, some of the univalents, which lie at the equator along with the other trivalents and bivalents, divide at the first division and the daughter halves go to opposite poles. In certain cells univalents are left out on the spindle while the others have moved to the poles. Lagging univalents sometimes divide late on the spindle and the split halves may be gathered into the same daughter nucleus (Fig. 27). In Fig. 28 three bivalents are found lagging on the spindle. This is a very rare condition. Very irregular anaphases observed are illustrated in Figs. 25, 27, and 29. The two daughter groups of chromosomes in Figs. 23 and 24 show more than the  $2n$  number, which obviously has been caused by the division of the univalents at anaphase I. The two groups in Fig. 23 are slightly fused. Fig. 37 repre-

sents a first telophase and two chromosomes are left out. Chromosome elimination at the first division has been observed frequently.

Irregularities at anaphase I frequently led to the formation of restitution nuclei (Figs. 31, 32, and 33). While this has been reported in triploid hybrids,



FIGS. 23-37. All figures were drawn with a camera lucida at bench level. An achromatic objective N.A.1.3 was used in conjunction with an  $\times 30$  ocular, giving an initial magnification of 5,400 diameters. The figures have been reduced to a half in reproduction. Fig. 23. Two anaphase I groups nearly merged. Fig. 24. Anaphase I. Both the groups show seventeen units due to division of the univalents at the first division. Fig. 25. A very irregular anaphase I. Fig. 26. Anaphase I. Two lagging univalents which have divided. Fig. 27. Anaphase I. The division is interrupted and there are more than the diploid number of bodies. Fig. 28. Anaphase I. Three lagging bivalents. Fig. 29. Anaphase I. Very irregular division and the total number is more than twenty-seven. Fig. 30. Anaphase I. Four dividing univalents. Figs. 31-33. Irregular anaphases leading to a restitution nucleus. Figs. 34-36. Anaphase I. Bridges and fragments. The fragments are of variable sizes. Fig. 37. Telophase I. Two chromosomes are not included in the cell.

the causes underlying their formation are not understood. One of the consequences of this aberration is the production of unreduced gametes. It has been reported in *Hieracium* (Rosenberg, 1917, 1926, 1927); *Balanophora* (Kuwada, 1928); *Triticum* (Kagawa, 1929); *Digitalis* (Buxton and Newton, 1928); *Prunus* (Darlington, 1930); *Narcissus* (Nagao, 1929); *Oryza* (Ramanujam, 1937). Restitution nuclei are organized at the second division also in the present material, as is obvious from Figs. 52, 53, and 54. In Fig. 54 the two groups at the bottom of the cell are merged while the two at the top are separate, one being in lower focus.

In spite of frequent lagging of chromosomes at the end of the first division, only two nuclei are formed, the laggards obviously being gathered up in the daughter nuclei. In the distribution of chromosomes to the daughter cells in a triploid, there is the ordinary separation of the chromosomes of the two genomes and the random segregation of the third set, so that gametes are produced with an approximately binomial frequency distribution of chromosomes, ranging from the haploid to the diploid number. This has been found to be true in most triploids where there is not an excessive elimination of chromosomes due to lagging. As examples we have: *Hyacinthus* (Belling, 1924); *Tradescantia* (Darlington, 1929); *Allium* (Levan, 1935); *Oenothera* (Capinpin, 1933); *Petunia* (Dermen, 1931); *Canna* (Belling, 1921); *Disporum* (Hasegawa, 1933); *Petunia* (Steere, 1932); *Nicotiana* (Lammerts, 1929); *Datura* (Belling and Blakeslee, 1922). This type of assortment may be upset either by excessive lagging and elimination of chromosomes (*Narcissus*, Nagao, 1935) or by irregular disjunction of multivalents (cotton, Skovsted, 1934). In the triploid *Calceolaria* studied, the high frequency of trivalents does not seem to interfere with the law of binomial frequency distribution.

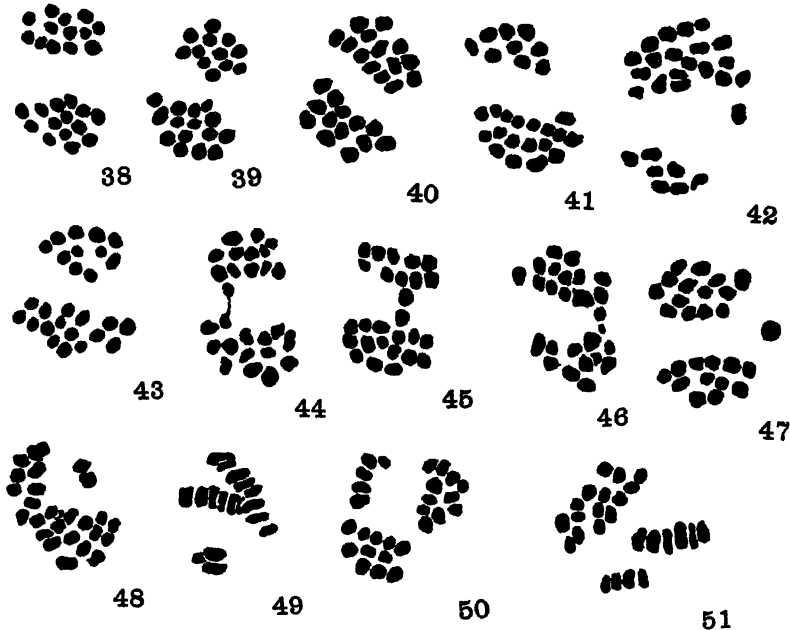
At first anaphase the separation of one pair of chromosomes is sometimes not clean, leading to the formation of a chromatin bridge and fragment. Similar configurations have been observed by McClintock (1931a) in maize, Müntzing (1934) in *Crepis*. This indicates that the plant is heterozygous for an inversion. A cross-over in the inverted segment produces a chromatid with two centromeres and one without any. The fragment usually lies a little away from the bridge and it varies in size (Figs. 34, 35, and 36). If the chromatids concerned in the inversion do not separate at the first division, chromatin bridges appear at second anaphase. This is due to the presence, in addition to the chiasma in the inverted region, of a second formed proximal to the inversion chiasma, involving one chromatid crossing over at both chiasmata and leading to the loop type of configuration (Smith, 1935) (Figs. 55 and 56). Fig. 57 shows two bridges at second telophase, the result of two chiasmata, one in the inversion and the other proximal to it, the chiasmata being asymmetrical, i.e. the chromatid relationship altered by the first is not restored by the second. The occurrence of more than one bridge indicates the formation of a proximal chiasma disparate to the inversion chiasma in more than one bivalent. In this case only one fragment has survived the first division. A univalent bridge at second division is illustrated in Fig. 58. The other two daughter groups have formed a restitution nucleus. The causes leading to the formation of a univalent bridge will be examined later. A univalent bridge at second division has not been reported previously.

The second division is very regular, the univalents obviously dividing on the homotypic spindle along with the other chromosomes. The divided halves of the univalents derived from the first division do not divide again, but are distributed at random to the poles. The separation of chromosomes at metaphase II is generally 14-13; other variations are indicated in the table.

TABLE I

Chromosome partition.	20+7.	17+10.	16+11.	15+12.	14+13.	Total.
Nos. of cells formed	1	1	2	5	26	35
Percentage	2	2	5	14	75	100

Figs. 38-43 illustrate the different types of distribution at metaphase II. Belling (1921) found a similar frequency of distribution in triploid *Canna*,



FIGS. 38-51. All figures were drawn with a camera lucida at bench level. An achromatic objective N.A.1.3 was used in conjunction with an  $\times 30$  ocular, giving an initial magnification of 5,400 diameters. The figures have been reduced to a half in reproduction. Fig. 38. Metaphase II. 14-13. Fig. 39. Metaphase II. 15-12. Fig. 40. Metaphase II. 14-13. Fig. 41. Metaphase II. 18-9. Fig. 42. Metaphase II. 20-7. Fig. 43. Metaphase II. 16-11. Fig. 44. Two groups at metaphase II with a chromatid bridge. Figs. 45-6. Metaphase II. The two groups in each case are merged. Fig. 47. Metaphase II. One chromosome is isolated from the two groups. Fig. 48. Metaphase II. Two chromosomes are isolated. Fig. 49. Metaphase II. Three chromosomes are isolated. Fig. 50. Metaphase II. Five chromosomes are isolated. Fig. 51. Metaphase II. Four chromosomes are isolated.

the majority of the cells showing a 14-13 separation. An unequal distribution at metaphase II would obviously lead to the formation of gametes with variable numbers of chromosomes. This disparity is no doubt caused by irregular segregation at anaphase I. One interesting feature in the triploid studied, is that out of the many cells counted at metaphase II, not even one had more than the  $2n$  number, indicating that there was no double division of the univalents. In apomictic forms like *Hieracium pseudophyllum* (Rosenberg, 1927), however, an equational splitting of all the chromosomes at both

the divisions resulted in diploid gametes. There are a few recorded cases where some or all of the univalents divided twice in meiosis: *Viola* (Clausen, 1926); *Brassica* (Morinaga, 1929); *Raphano-Brassica* (Karpechenko, 1927, 1928); *Nicotiana* (Müntzing, 1935). It is doubtful whether the diploid gametes thus formed are functional, excepting the case of *Pygaera* (Federley, 1928).

There is a frequent elimination of chromosomes at metaphase II, sometimes as many as five chromosomes being left out (Figs. 47-51). These are not organized into a micronucleus. Elimination of chromosomes at this stage is not unknown. Avery (1929) found in trisomics of *N. alata* var. *grandiflora* that over 10 per cent. of 571 pollen mother-cells examined had two to five chromosomes in the plasma. Müntzing (1935) found a similar condition in *Nicotiana* hybrids. He suggests that gametes with deviating numbers are produced by both irregular disjunction of multivalents and non-conjunction, a view also shared by Avery (1929). Elimination and loss of chromosomes has been reported in *Nasturtium* (Manton, 1935); *Nicotiana* hybrids (Goodspeed and Clausen, 1922); and triploid potatoes (Rybin, 1930). Variable numbers at metaphase II have been recorded by Müntzing (1933) in triploid *Solanum tuberosum*. In *Brassica* hybrids (Morinaga, 1929), eleven and eighteen chromosomes were often found at metaphase II, although the usual number was fourteen to fifteen. But there was good agreement of the observed numbers with the frequency calculated on the assumption of a chance distribution of the univalents. Another circumstance which might lead to irregular numbers at metaphase II may be the failure of the multivalents to separate at anaphase I. In triploid rice, according to Ramanujam (1937) some cells had twelve trivalents ( $2n = 36$ ) distributed on the spindle without disjunction of the components, and in one case he found five trivalents moving towards one pole and seven trivalents towards another. He assumed that in these cases the chromosomes of the trivalents were so closely compacted together that they did not disjoin in the first division but came out again as such in the second.

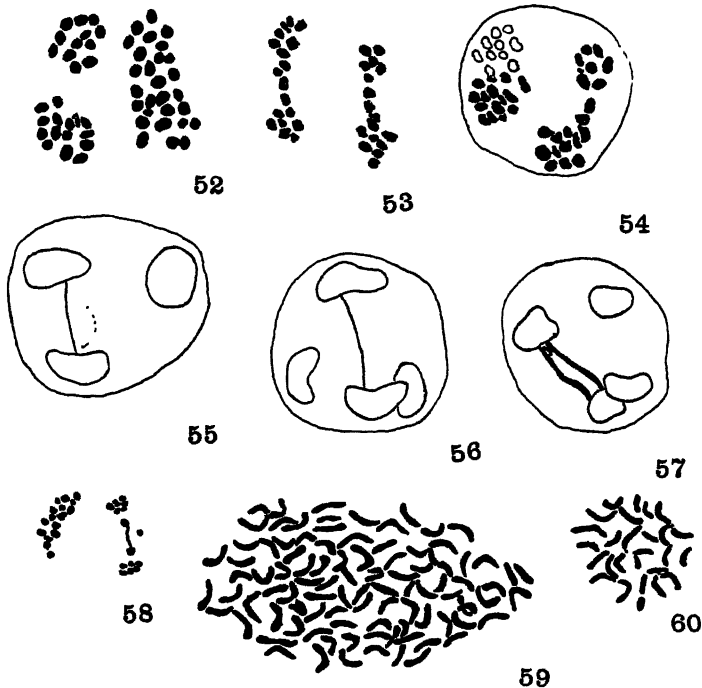
At metaphase II, the two groups of chromosomes are frequently bridged (Fig. 44), and in some they are more or less merged (Fig. 46). A similar linking up of metaphase groups has been observed by Müntzing in *Nicotiana* hybrids (1935) and triploid *Solanum tuberosum* (1933). If two metaphase groups merge, this would undoubtedly lead to the formation of a dyad.

#### IV. DISCUSSION

##### (a) Chromosome pairing.

The behaviour of the chromosomes during meiosis in triploids has been the subject of unabated interest owing to the presence of three sets. The zygotene is undoubtedly the crucial stage and has been studied with particular care in many triploids, in order to interpret the mode of pairing. On the chiasma theory of pairing (Darlington, 1929), the chromosomes at this stage

are very long and straggle all round the nucleus. Pairing usually begins at several isolated points where homologous parts of chromosomes accidentally come into close proximity, and extends along the chromosome from these points. Chiasmata are formed at random only between those parts of chromo-



FIGS. 52-60. All figures were drawn with a camera lucida at bench level. An achromatic objective N.A.1.3 was used in conjunction with an  $\times 30$  ocular, giving an initial magnification of 5,400 diameters. The figures have been reduced to a half in reproduction. Fig. 52. Anaphase II. The two groups on one side are merged. Fig. 53. Early telophase II. The two groups on either side are merged. Fig. 54. Anaphase II. The two groups on the lower side are merged. The two upper groups are in different foci and separate. Figs. 55-6. Telophase II. Chromatid bridge. The fragment has been lost in both cases. Fig. 57. Telophase II. Double bridge. Only one fragment is found. Fig. 58. Telophase II. A univalent bridge and a small fragment is found between the groups on the right, and the groups on the left are merged. Fig. 59. A somatic cell from the wall of the anther with more than one hundred chromosomes. Fig. 60. Somatic metaphase.  $2n = 27$ . Three satellited chromosomes.

somes that are paired at pachytene, the mean chiasma frequency being proportional to the lengths paired (Darlington, 1931). As prophase proceeds, the forces of repulsion acting between the attachment constrictions compel the chiasmata to travel towards the distal ends of the chromosomes, resulting in a reduction of the number of chiasmata by telescoping them at the chromosome ends to form terminal chiasmata. The shape of each configuration at diakinesis is determined by the number and position of the chiasmata present in conjunction with the position of the centromere on the paired chromosomes.



The burden of Darlington's argument is twofold: (i) that only homologous parts of the chromosomes are paired at pachytene, and (ii) that chiasmata form only between those parts of the chromosomes that are paired at pachytene. A number of alternative explanations for the origin of chiasmata have been given. Wenrich (1916) believed that chiasmata arose by the random formation of loops in four associated threads. Belling (1923) sought to explain the origin of chiasmata on the view that crossing-over depended on coincidence of fortuitously occurring breaks in all four chromatids, combined with a movement of torsion and reunion of the newly opposed threads.

Darlington's hypothesis that only homologous parts of chromosomes pair at pachytene has been subjected to severe criticism. Gates (1908) found in a triploid *Oenothera* ten and eleven chromosomes moving regularly to the poles at reduction division as if non-homologous chromosomes were paired. Yarnel (1931) found in triploids of *Fragaria* ( $2n = 21$ ) that there were three associations of four chromosomes, one trivalent and three pairs, and explained it as due to pairing of non-homologous chromosomes. McClintock (1933) has shown in *Zea Mays* that non-homologous parts of chromosomes can pair at zygotene after the homologous portions are paired. She states that pairing between non-homologous parts was as intimate as between homologous portions, but found no evidence for the existence of such pairing by chiasma formation up to diakinesis. Burnham (1934) arrives at similar conclusions from a study of interchange heterozygotes in maize, but Lammerts (1934) not only finds non-homologous parts paired at pachytene in a haploid *Nicotiana*, but advances evidence in support of the view that such pairing can and often does persist until metaphase by the formation of chiasmata between the paired non-homologous parts. He concludes that this type of pairing might be common in haploids and species hybrids.

A great many triploids have been examined cytologically in order to elucidate the nature of pairing, which in turn leads to the observed configurations at diakinesis. Complete trivalency has been reported in *Canna* (Belling, 1921, 1925); *Datura* (Belling and Blakeslee, 1922, 1923); *Hemerocallis* (Belling, 1925); *Hyacinthus* (Belling, 1923, 1929); *Narcissus* (Nagao, 1929); *Thea* (Karasawa, 1932); *Petunia* (Steere, 1932); *Lycoris* (Inariyama, 1931); *Disporum* (Hasegawa, 1933). While complete trivalency is usual, sometimes meiotic irregularities lead to disruption of the trivalents into bivalents and univalents. All the triploids mentioned above are autotriploids. But in triploids of *Solanum* (Lesley, 1928), *Zea* (McClintock, 1929), *Morus* (Osawa, 1920), *Campanula* (Gairdner, 1926), *Pyrus* (Darlington and Moffet, 1930), *Aconitum* (Afify, 1933), *Primula* (Dark, 1931), *Hemerocallis* (Dark, 1932), *Tulipa* (Newton and Darlington, 1929), *Solanum* (Janaki Ammal, 1934), *Nicotiana* (East, 1933), *Fritillaria* (Darlington, 1936), and *Oryza* (Morinaga and Fukushima, 1935), varying numbers of trivalents, bivalents, and univalents are formed. Apparently, then, the complete association of homologous chromosomes in triploids is rarely possible.

The greatest possible deviation from the autotriploid type is shown by those plants which form no trivalents at all but have only bivalents and univalents. Most of these are hybrids which have acquired two sets of chromosomes from one parent and one set from another, the two parents belonging to different species or even genera. Under such circumstances, there can be very little association of chromosomes into trivalents, since they are only slightly or not at all homologous. The earliest and best known examples were the *Drosera* hybrids, secured by crossing *D. rotundifolia* ( $n = 10$ ) with *D. longifolia* ( $n = 20$ ) (Rosenberg, 1909). Rosenberg found no trivalents at all but ten paired and ten unpaired chromosomes. Similar behaviour has been reported since, in several species hybrids, *Rosa* (Erlanson, 1929), *Triticum* (Kihara, 1924; Sax, 1922; Thompson, 1926), *Nasturtium* (Manton, 1935), *Oryza* (Ramanujam, 1937). Two sets from one of the parents pair off together, leaving the remaining set from the other parent isolated as univalents. It is possible that, in hybrids, genetic homology of the chromosomes alone is not sufficient for chromosome pairing. Hollingshead (1930) found in a diploid *Crepis capillaris* which had arisen by doubling in a haploid, all gradations from full pairing to all chromosomes univalent. That different nutritional conditions in the cells of the anther may influence conjugation is a suggestion from Meurman (1928), who found in *Ribes gordonianum* certain cells of smaller size lying in the middle of the anther showing full conjugation, while others of greater size near the periphery had all univalents.

Katayama (1931) and Stow (1926, 1927) claim that temperature affects conjugation to a considerable degree. In the potato, temperature above 25–30° C. appeared to inhibit chromosome conjugation. Heilborn (1930) found in apple varieties all the meiotic chromosomes appearing as univalents when subjected to temperature of 35° C. Sax (1931) has produced temporary asynapsis in *Rhoeo discolor* by subjecting plants to low temperature. Mather (1935) found a diurnal variation in chiasma formation in wheats.

Genetic factors may influence conjugation of chromosomes. Crossing-over is sometimes prevented by a gene, as in *Drosophila* (Gowen, 1928), and this in turn affects pairing. Specific genes controlling the conjugation of chromosomes are known in *Zea* (Beadle, 1930), *Datura* (Bergner, 1934), *Oryza* (Ramanujam and Parthasarathy, 1935). Asynapsis due to mutation has been found in *Primula* (Newton and Pellew, 1929), *Viola* (Clausen, J., 1930), *Nicotiana* (Clausen, R. E., 1931; Avery, 1929), *Sorghum* (Huskins and Smith, 1934), *Avena* and *Triticum* (Huskins and Hearne, 1933).

From the foregoing it is obvious that in triploids there is a gradual transition between complete associations of all the chromosomes into trivalents and the complete absence of chromosome aggregation. Darlington (1929) found in *Hyacinthus* that homology is not the only factor in the formation of trivalents, but that size is also an important factor, since short chromosomes form trivalents much less frequently than do long ones. Manton (1935) found in *Nasturtium* that pairing sometimes fails completely, all the chromosomes

appearing as univalents. But there is no reference in the literature to an allotriploid where both these extremes appear in the same plant. Unfortunately, I have no means of verifying the horticultural history of this triploid *Calceolaria*, but its allopolyploid nature is obvious from the somatic complement and the heteromorphic nature of the satellites.

The high frequency of trivalent formation in this hybrid triploid is possibly due to the fact that it is a cross between closely related species followed by structural changes in the chromosomes. That pairing in the hybrid is a measure of taxonomic relationship between the parents is an established general principle. It has been found to be true in *Nicotiana* (Goodspeed, 1934), *Crepis* (Babcock and Emsweller, 1936). There are exceptions to this principle, such as Clausen's *Viola* hybrids (1931), *Nicotiana digluta* (Müntzing, 1932) and amphidiploid wheat-rye hybrids (Lewitsky and Benetzkaja, 1929, 1931). Mather (1935) found in a triploid wheat hybrid (*T. dicoccum* × *monococcum*) two constant morphological types of trivalents appearing at diakinesis. His explanation for this constancy and high frequency of trivalent formation is that it is the result of autosyndetic pairing between two *dicoccum* and one *monococcum* chromosomes. He discards the idea of structural changes in chromosomes as being responsible for this high frequency of trivalents, on the ground that more than two trivalents must appear if that were so. I have not been able to observe any morphological constancy in the types of trivalents. It seems reasonable to assume that in this hybrid the high frequency of trivalent formation is due to the fact that the parent of the autotetraploid species was nearly related to the diploid species in the cross.

In his work on triploid *Solanum tuberosum*, Müntzing (1933) says: 'In triploid *tuberosum* varieties investigated by me a high proportion of the chromosomes are united to trivalents at diakinesis and first metaphase. This indicates autotriploidy'. The allotriploid described in this paper with its high frequency of trivalent formation, provides a contradiction to Müntzing's statement.

Most triploids are sterile on account of their unstable chromosome complex. Sexual reproduction would lead to irregular segregation and extinction of the type. But certain triploid species and varieties are able to maintain their chromosome complex and consequent taxonomic individuality by various types of apomixis. Rosenberg (1917) found that a constant wild species of *Hieracium*, *H. umbellatum*, is triploid, and that the embryos, in the seeds which produce triploids, arise from nucellar buds. Holmgren (1919) found similar phenomena in various naturally occurring species of *Eupatorium* and *Erigeron*. Most triploids of this type preserve their triple set of chromosomes by more obvious means of vegetative reproduction. Typical triploid species and varieties of this kind have been found in a large number of genera which multiply by bulbs, stolons, rhizomes, &c. They have been described in *Iris* (Kazao, 1929), *Rubus* (Longley, 1924), *Rosa* (Erlanson, 1929), *Tulipa* (Newton and Darlington, 1929), *Hyacinthus* (Belling, 1925), *Narcissus*

(Nagao, 1929), *Canna* (Belling, 1921, 1925; Tokugawa and Kuwada, 1924), *Hemerocallis* (Belling, 1925), *Fragaria* (Yarnel, 1929) and others. In general these triploids have appeared spontaneously either in nature or under cultivation and are probably the result of natural or artificial hybridization. Darlington (1937) thinks that this type of clonal reproduction is conditioned both by the sterility of the hybrid and by its particular genotype. However, vegetative reproduction provides the means of preserving the unbalanced chromosome complex in these hybrids. Such hybrids of *Calceolaria* are mostly propagated by vegetative means and may be added to the list of similar triploids (Srinath 1939).

(b) *Structural hybridity.*

Structural changes between homologous chromosomes will have different effects at metaphase and anaphase. These effects vary, not only in relation to the kind of structural change, but also with the cross-over relationships of chromatids in the whole bivalent. We may distinguish the following characteristic changes: (1) simple inversion of a segment, (2) translocation or duplication of segments which come to occupy new positions in the chromosome, which are 'straight' or 'inverted' in relation to their original directions within the chromosome.

An inversion has been defined as the reversal of the linear sequence of the 'genes' in one segment of a chromosome relative to the centromere (Sturtevant, 1926). It can arise by breakage of a chromosome at the locus of crossing of a loop and a different type of reunion of the ends.

The presence of an inverted segment can be inferred at pachytene by the disturbance it causes to normal pairing, at anaphase I and II where they result in dicentric chromatid bridges and acentric fragments. The significance of the occurrence of the bridge and fragment was first inferred by McClintock (1933) in irradiated maize as cytological evidence for crossing-over in the inverted segment. Smith (1936) in *Trillium*, found a loop chromatid in the first anaphase, in addition to the bridge and fragment, the former giving a bridge at the second division due to the split of the centromere. Inverted segments due to irradiation have been described in *Drosophila* (Painter and Stone, 1935; Grüneberg, 1935). They may also arise as a result of hybridization between different species, as in *Crepis* (Müntzing, 1934), *Triticum* (Mather, 1935), *Lilium* (Richardson, 1936), *Nicotiana* (Müntzing, 1935), *Zea* × *Euchlaena* (Beadle, 1933), *Hemizonia* (J. Clausen, quoted by Müntzing, 1935). Bridges are also obvious from the illustrations of meiosis in *Festuca* × *Lolium* (Peto, 1933), *Avena* (Nishiyama, 1929), although they were not reported as such at that time. Evidence of the occurrence of inverted segments in natural populations has also been found in species of various plants and animals: *Trillium* (Smith, 1935), *Tulipa* (Upcott, 1937), *Chorthippus*, *Stauroderus* and *Tradescantia* (Darlington, 1936*b* and 1937*a*). Inversions are used to separate natural races in *Drosophila*. (Sturtevant and Dobzhansky, 1936; Koller, 1935).

The formation of a bridge and fragment is dependent on the dimensions of the relatively inverted segment. If it is too small to allow a cross-over, then a bridge and fragment are not formed. In such cases there will be a non-homologous association (McClintock, 1933) in the inverted zone but no cross-over occurs. Since the inversion lies between two homologous pairing segments, it is subjected to torsion pairing with the normal segment.

A dicentric bridge and an acentric fragment at anaphase I can arise under the following circumstances: (1) a single chiasma in the inversion, (2) two cross-overs in the inversion, in which one chromatid is involved in both the cross-overs, (3) one cross-over in the inversion and another proximal to it, the two chiasmata being symmetrical.

A bridge and fragment at anaphase II is due to two cross-overs, one in the inversion and one in the region proximal to it, in which only one chromatid is involved in both cross-overs. A monocentric loop and a fragment would be formed at anaphase I. The loop chromatid forms a bridge at anaphase II, the centromere having divided. The fragment may or may not survive the first division. The occurrence of more than one bridge at the second division indicates the formation of a proximal chiasma disparate to the inversion chiasma in more than one bivalent.

As a rule, bridges formed at first division break during telophase when the strain of the separating centromeres becomes too great. As the centromeres separate during anaphase the chromatid joining them is pulled out and becomes thinner. In the case of long bridges, the stretching does not begin until late anaphase. Here the centromeres can separate to their full extent without causing the bridge to break. Then the bridge survives till the second division. Such bridges have been occasionally seen at anaphase II diametrically uniting nuclei derived from different second meiotic divisions. They may distort and prevent the formation of the cell wall where they come into contact. They have been observed to undergo uncoiling during telophase at the same time as the chromosomes included in the nuclei (Trillium, Smith, 1935; Lilium, Richardson, 1936). Emsweller and Jones (1938) maintain that the dissolution of the bridge is due to enzymes in the cytoplasm. The acentric fragment is entirely passive. It may either lie free on the plate or be 'strangled' by the bridge chromatid coiling round it. Such a fragment may be carried into the nucleus, and has been seen lagging in the pollen grain mitosis in *Podophyllum* (Darlington, 1936b).

The size of the acentric fragment is a fair measure for judging the size of the inversion. Sax (1937) considers that fragments of different sizes may arise from the same inversion, either by the occurrence of inverted cross-overs in non-homologous straight association of inverted parts, or by crossing-over in loop pairing, which may shift throughout the length of the chromosome arm, extending to non-homologous regions as well.

The behaviour of inversions in triploids is complicated by certain factors, amongst which non-disjunction and precocious division of some of the centro-

meres may be mentioned. If the separation takes place without any coiling the dicentric chromatid will remain unbroken until the pollen grain mitosis. Non-disjunction, therefore, reduces the bridge frequency at the first division and increases it at the second. When we find a second division bridge in a triploid, this might have as much resulted by non-disjunction as by two chiasmata, one in the inversion and another proximal to it.

Univalent bridges arise from lagging members of trivalents (or multivalents), which have formed a chiasma in the inversion and one proximal to it, with one of their partners, and since they are parts of trivalents, they must have formed at least one chiasma with the other partner. During the post-pachytene stages up to anaphase one of its arms forms a closed loop. At anaphase its centromere, which is lagging on the equator, divides at the first instead of at the second division. The arm which was a loop then forms a bridge. But if the centromere divides at the second division the loop will form a bridge at the second division. In order that a univalent bridge shall be formed, the chromosome which has crossed over and formed chiasmata with its partners must be left on the plate at anaphase. Direct observation and statistical inference have shown that such a course of events is quite common in the separation of trivalents (Tulipa, Upcott, 1937). Upcott suggests that univalent bridges are rare and occur only in triploids with a high inversion frequency. Trivalents do not always give rise to lagging chromosomes with a loop (which gives rise to a bridge). This depends mainly upon their orientation at metaphase, which in turn depends upon the number and position of their chiasmata. Lagging members may arise when the co-orientation of the trivalents is linear or indifferent (cf. Darlington, 1936, 1937). It will be noticed that the conditions which lead to a univalent bridge due to the precocious split of the centromere are the same as those that would give a second division bridge by the chromatids of a bivalent. A univalent bridge is readily distinguishable from a bivalent bridge on account of its being made up of only two chromatids, and it will not join the poles.

The inversion frequency in inversion heterozygotes is often found to be not commensurate with the amount of structural change. This may be caused by two factors. It has already been pointed out that where the inverted segment is not large enough, a cross-over cannot take place. Apart from this purely mechanical difficulty the inverted segment may suppress crossing-over, as has been shown in certain cases. The genetical suppression of crossing-over in certain regions of the X-chromosomes in *Drosophila* has been found to be due to inverted segments (Sturtevant and Dobzhansky, 1936). The inversion may pair straight at pachytene, non-homologous parts associating by torsion (McClintock, 1933, on *Zea*; Darlington, 1936, on *Chorthippus*), but no cross-over is formed. All the 'genes' within the segment are prevented from crossing-over effectively with the 'genes' in the unchanged segment. It is, therefore, isolated in evolution.

It was pointed out earlier that most triploids are sterile and that they

maintain their triploid complex by methods of reproduction other than sexual. Where an organism leads a clonal existence over long periods of time, structural changes can accumulate. This is illustrated by the extreme heterozygosity of old clones (*Prunus* and *Rubus*, Crane and Lawrence, 1934), triploid garden tulips (Upcott and La Cour, 1936). A large number of structural differences is associated with sterility, which itself favours their further accumulation.

(c) *Univalents.*

In a triploid a variable number of univalents lag upon the plate after the other chromosomes have reached the poles. These univalents may have been the result of failure of pairing or they may be members of trivalents or other multiple associations left upon the plate after the separation of their partners. Failure of pairing is caused by at least three factors. These have been discussed earlier. Univalents which lag on the plate may suffer one of several fates. They may either divide or remain undivided, and divided or undivided they may be included in one of the daughter nuclei or remain lagging on the plate. The univalents either lie on the equator or off it. They may divide at the first division and pass more slowly to the poles during anaphase than do the halves of the bivalents. Richardson (1936) thinks that this indicates their weaker centromere charge. If they are not included in the daughter nuclei they usually degenerate.

## V. SUMMARY

1. Meiosis is described in a triploid *Calceolaria* hybrid ( $2n = 27$ ).
2. In polar views of somatic metaphase three chromosomes have satellites. The heteromorphic nature of the sat-chromosomes points to the hybridity of the plant.
3. From nought to nine trivalents occur at diakinesis and metaphase of division I, the most frequent configuration being  $6_{II} + 5_{III}$ . The frequency of trivalent formation is very high, which is a rare condition in an allotriploid.
4. The univalents mostly divide at the second division, lagging chromosomes being rare. A very small loss of chromosomes occurs at anaphase I, and a much greater loss at the second division.
5. The first anaphase is very irregular, and leads to the formation of restitution nuclei. The same irregularity was observed at the second division.
6. The presence of inverted segments is indicated by the appearance of chromatin bridges at anaphases I and II.
7. A univalent bridge at the second division is described. This is rare and has not been previously reported.
8. The separation at metaphase II is very variable, ranging from 14-13 to 18-9.

# VI. ACKNOWLEDGEMENTS

I wish to acknowledge my deep indebtedness to Professor R. R. Gates for his guidance, criticism, and encouragement during the progress of this work.

I have to thank the Government of India for the award of a foreign scholarship, the holding of which has enabled this study to be undertaken.

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# The Effect of Cyanide on the Respiration of Barley

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With nine Figures in the Text

THE experiments described in this paper were designed as a first step towards discovering the nature of the oxidizing systems effective in barley respiration. They made clear its degree of sensitivity to cyanide and gave some indication of where the sensitivity lies.

## MATERIAL AND METHODS

Barley, var. Plumage Archer, was used throughout, and supplied by Messrs. Suttons after the harvests of 1935 and 1936. The grain was stored in a dry bin and the most recent supply was always used. The majority of the experiments were performed with leaves cut from young plants. Batches of leaves were enclosed in dark, cylindrical chambers, through which CO<sub>2</sub>-free air was passed, and allowed to take up the desired solutions through their cut ends. This method of infiltration had already been employed and found successful in other experiments, in spite of the poor transpiration conditions involved (see James and Norval, 1938). The gas stream passing through the leaf chamber was collected over mercury and analysed for oxygen and carbon-dioxide by the Haldane 'constant pressure' method, exactly as described by Haldane and Graham (1935). The collection of the gas was automatic and provided samples for each successive two-hour period. The apparatus was twinned, so that two experiments, with and without cyanide, could be run simultaneously. Full details of the collection apparatus are being given elsewhere (James and James; in preparation).

## RESULTS

*M/500 cyanide.* Barley of the 1935 harvest was germinated in February 1936 and grown on for ten days in a greenhouse. Each plant had then one fully expanded green leaf. One hundred were cut off and divided into two lots of fifty and each sample placed at once in a darkened glass cylinder just large enough to hold it comfortably. The cut leaf bases dipped into solutions

about an inch deep in the bottom of the chambers. The first solution consisted of M/500 KCN (B.D.H. analar) acidified with a few drops of strong HCl to pH 4.6: the second was distilled water similarly acidified. The chambers were closed and connected with the gas moving and collecting apparatus. The first gas collection, 0–2 hours, was rejected, and the succeeding collections analysed up to 50 hours. The results are shown in Fig. 1.

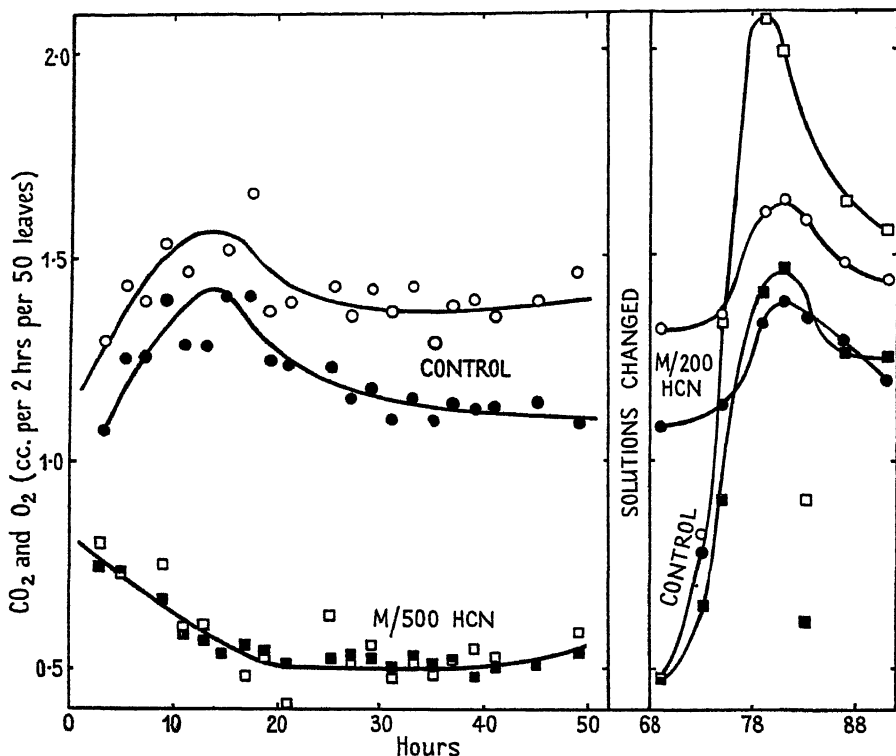


FIG. 1. ○—○ Oxygen; leaves receiving water only for the first 50 hours and M/200 HCN later. ●—● Carbon dioxide; leaves receiving water only for the first 50 hours and M/200 HCN later. □—□ Oxygen; leaves receiving M/500 HCN for the first 50 hours, but water only after 68 hours. ■—■ Carbon dioxide; leaves receiving M/500 HCN for first 50 hours, but water only after 68 hours.

After 50 hours the experiment was interrupted. The solutions were washed out of the leaf chambers and the bases of the leaves also washed carefully with distilled water. The sample which had hitherto received cyanide was set up again with distilled water and HCl to pH 4.6 and the previous control sample was put in M/200 KCN with HCl to pH 4.6. The apparatus was set up again and collection resumed 68 hours from the beginning of the experiment. During the interval between 50 and 68 hours the leaves were in the dark, except for diffuse light while the solutions were being changed. The results are shown in the second part of Fig. 1. Both lots of leaves, when

finally taken from the apparatus, had begun to yellow but were still turgid and healthy. They had no smell.

Both oxygen absorption and carbon-dioxide formation were rapidly depressed by the cyanide; oxygen absorption fell to little more than a third of the control value, and carbon-dioxide emission to about one-half. The respiratory quotient (RQ) was consequently raised, as shown in Fig. 2. The

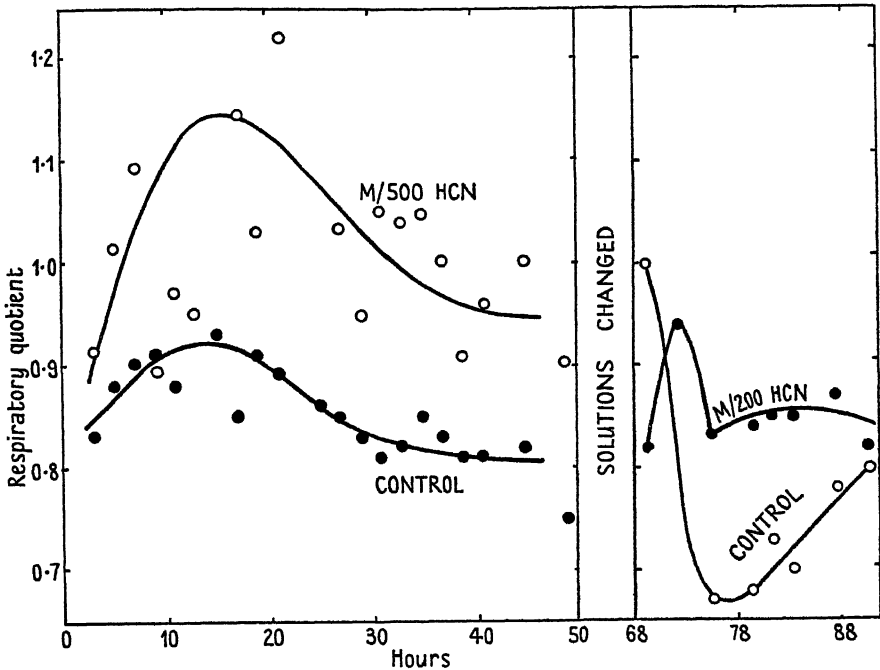


FIG. 2. Respiratory quotients: ○—○ Leaves receiving M/500 cyanide for the first 50 hours and water only after 68 hours. ●—● Leaves receiving water only at first and M/200 HCN later.

irregularities in the cyanide curve are due to errors in determining the very small quantities of oxygen. After removal of the cyanide the oxygen absorption and carbon-dioxide formation rapidly rose, and the RQ fell. The effect of M/500 cyanide is, therefore, fully and easily reversible. The result of applying M/200 cyanide as late as the 68th hour is not so simple at first sight. The gas exchanges do not at once fall below the rates shown by the same leaves just before the application of the cyanide. This is because the rates in normal experiments without cyanide show a marked rise (the 'respiration hump') at this time (cf. experiments 5 and 7; Fig. 6). In the present experiment it is noticeable that the cyanide values fall below those for the leaves from which cyanide has been removed. In addition, the cyanide RQ begins to rise and crosses over the other. A partial inhibition has, therefore, occurred, particularly in oxygen absorption, as in the first part of the experiment.



*M/150 cyanide.*

An experiment was set up as before. Both lots of leaves were at first provided with distilled water only. After 6 hours the set showing the faster respiration-rate was transferred to *M/150* KCN acidified with HCl to pH 4.2. The other set was given *M/150* KCl+HCl to pH 4.2. The experiment was

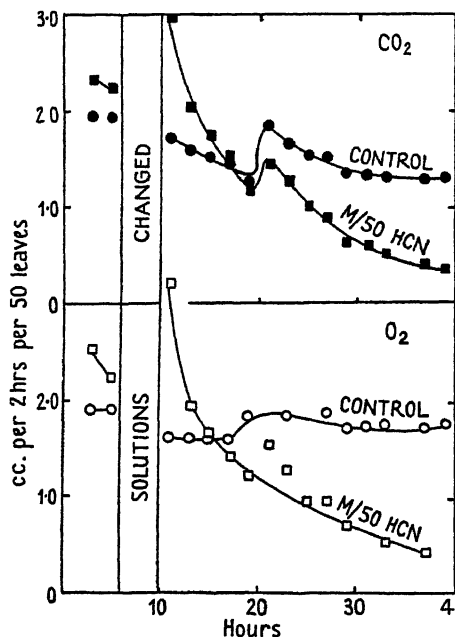


FIG. 3.

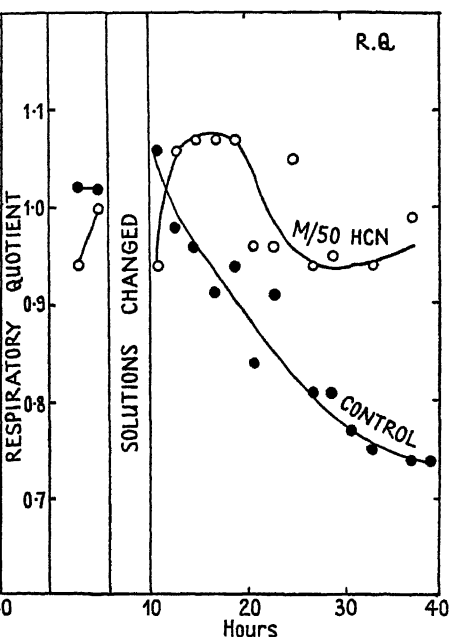


FIG. 4.

FIG. 3. ●—● Carbon dioxide; leaves receiving *M/150* KCl+HCl to pH 4.2. ■—■ Carbon dioxide; leaves receiving *M/150* KCN+HCl to pH 4.2. ○—○ Oxygen; leaves receiving *M/150* KCl+HCl to pH 4.2. □—□ Oxygen; leaves receiving *M/150* KCN+HCl to pH 4.2. For the first 6 hours all leaves received water only. FIG. 4. Respiratory quotients. ●—● Leaves with acidified KCl. ○—○ Leaves with acidified KCN. For the first 6 hours all leaves received water only.

then restarted and the gas exchanges followed for the next 30 hours. The results are shown in Figs. 3 and 4. The same effects are observable as in the previous experiment.

*M/100 and M/50 cyanide.*

A further experiment was carried out at *M/50*. Two batches of 60 leaves from 13-day-old plants were put into the respiration chambers. In the first the cut ends were in *M/50* KCN and in the second in *M/50* KCl. After 24 hours the cyanide solution was removed and replaced by *M/100* KCl, and the original chloride solution was replaced with *M/100* KCN. All the solutions were acidified to pH 4.2 with HCl. The results are shown in Fig. 5. Oxygen consumption is completely suppressed by *M/50* cyanide and there

is no recovery when it is replaced by chloride. Carbon-dioxide emission is suppressed nearly completely and also irreversibly. Application of M/100 cyanide after 24 hours' starvation also depresses the gas exchange, but does

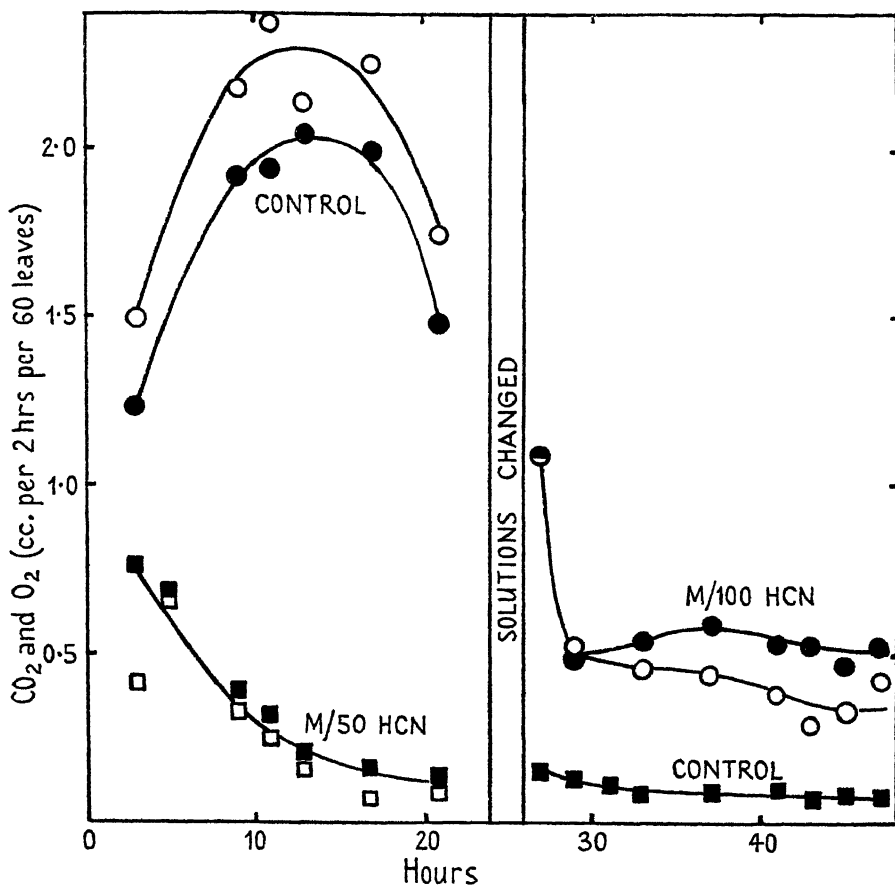


FIG. 5. ●—● Carbon-dioxide emission in presence of acidified M/50 KCl; and acidified M/100 KCN after 24 hours. ■—■ Carbon-dioxide emission in presence of acidified M/50 KCN; and water only after 24 hours. ○—○ Oxygen M/50 KCl acidified; acidified M/100 KCN after 24 hours. □—□ Oxygen M/50 KCN acidified.

not suppress oxygen consumption entirely. At the end of the experiment the leaves were giving off a sweet, hay-like smell, and there was no sign of putrefaction.

#### *The respiration curve under nitrogen.*

To help clarify the interpretation of the above results the following experiments were carried out with oxygen eliminated from the system. Since only carbon dioxide had to be estimated, the gas passing through the leaf chambers was bubbled through Pettenkofer tubes, and CO<sub>2</sub> estimation carried out by

the usual Pettenkofer method. The gas stream was automatically switched from one tube to the next every 6 hours by a mechanism which is being fully described in another paper (James and James; in preparation). Commercial compressed nitrogen was used, and the 2–3 per cent. of oxygen present removed by bubbling the gas stream through three successive Pettenkofer tubes containing alkaline pyrogallol. The gas stream was then allowed to pass into the plant chamber. The rate was controlled by a fine valve and kept approximately equal to the rate of flow of  $\text{CO}_2$ -free air through a control chamber.

Grain of the 1936 harvest was used for these experiments, and grown in a greenhouse as before. The first leaf was used when fully expanded and sixty such leaves taken for each sample. The leaves were cut off near the haulm and immediately put into the respiration chamber. Their cut ends dipped into a complete mineral solution (as in James and Norval, 1938) in both nitrogen and air chambers. The chambers were then closed and the gas stream started. The results of three experiments carried out in this way are assembled in Fig. 6. It appears that under the given experimental conditions the absence of oxygen does not greatly alter the rate of carbon-dioxide emission until the respiration rate in air begins to rise. Under nitrogen this rise is perceptibly reduced. Experiment 6 differs from experiments 5 and 7 in that the rise begins almost at once; this is characteristic of starved leaf material (cf. Yemm, 1934).

#### *M/500 cyanide under nitrogen.*

In these experiments nitrogen, purified as before, was passed through both plant chambers. The solution in one was M/500 potassium cyanide, and in the other M/500 potassium chloride. Both solutions were adjusted to pH 4.2 with a few drops of strong HCl. Mature leaves of plants grown in the field were used, the middle 6 inches of each leaf being taken. Each sample weighed 10 gm. Three experiments were carried out, and their results are shown in Fig. 7. It is clear that the HCN caused no appreciable depression of  $\text{CO}_2$ -output during the first 24 hours, but reduced it thereafter. This is in marked contrast with the experiments in air described above, in which carbon-dioxide output and oxygen absorption are depressed from the very start. The hump in the respiration curves without HCN is more marked in these experiments than in experiments 5, 6, and 7; this is due to the older leaves used. It is noteworthy (cf. p. 117) that the principal effect of HCN under nitrogen is to reduce this hump.

#### *Germinating embryos.*

The previous experiments were all carried out with fully expanded green leaves. Excised embryos have been found very interesting material for respiration work, and their responses to oxygen deficiency and dilute cyanide were also investigated. The technique of handling them is described fully in James and James (in preparation).

A sample of 1936 grain was soaked and 220 embryos excised; they were divided into two equal lots of 110 and placed upon germination pads of compressed cellulose saturated with a complete culture solution. The pads were placed in Petri dishes with an additional 15 ml. of solution and

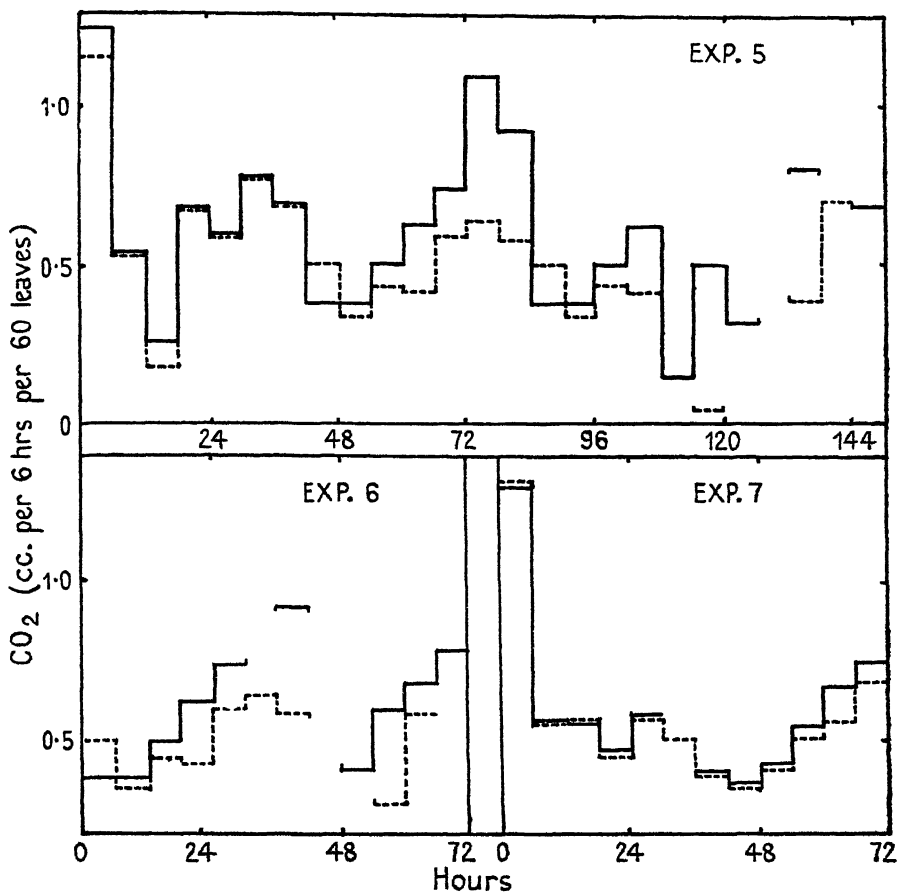


FIG. 6. Carbon dioxide (c.c./6 hr.) given out by 60 leaves — in air, - - - in nitrogen; in three independent experiments.

enclosed in domed respiration chambers of the type already described (James and Norval, 1938). Purified nitrogen was passed through one chamber and CO<sub>2</sub>-free air through the other. The CO<sub>2</sub> given off was collected in Pettenkofer tubes. After 48 hours the gas streams were changed over and the experiment continued 48 hours longer. At the end of the experiment the chambers were opened up and the embryos examined. That set which was originally placed in nitrogen showed very little sign of germination. There was evidence that the radicles were just about to burst through; the plumules were not visible. The set which had been placed at first in air

showed normal germination; the radicles averaged about 8 mm. long and the plumules 2 mm.

It will be noticed (Fig. 8) that the difference in carbon-dioxide output is at once well marked, in contrast with the similar experiments performed on leaves where no considerable differences were observed for 48 hours. The

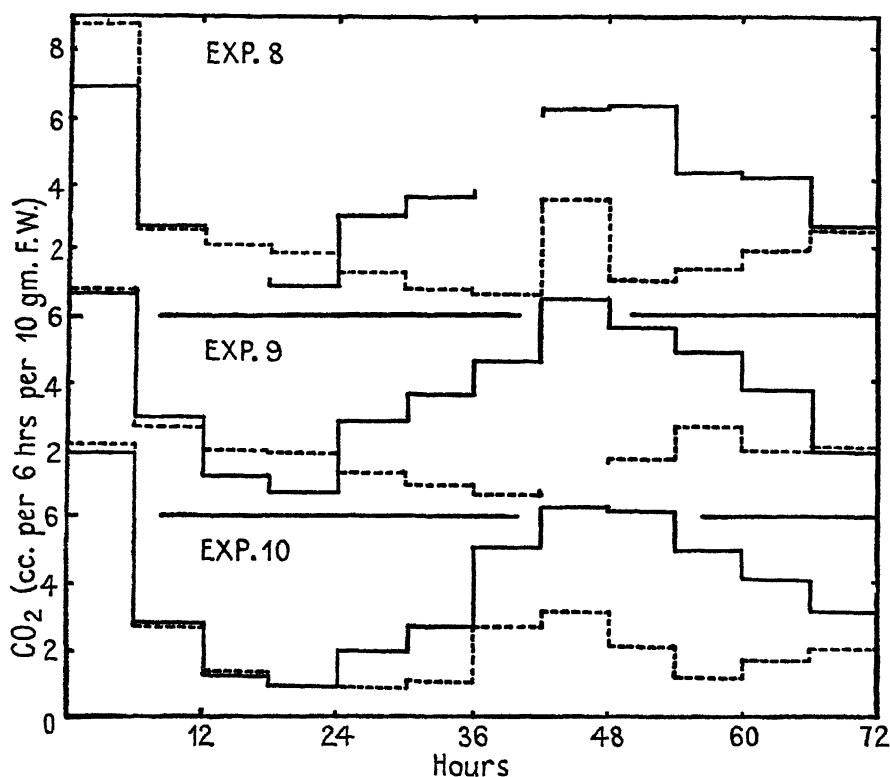


FIG. 7. Carbon-dioxide emission (c.c./6 hr.) of leaves under nitrogen. — leaves supplied with acidified M/500 KCl; --- leaves supplied with acidified M/500 KCN.

carbon-dioxide output of the embryos under nitrogen was rapidly reduced to an unmeasurable quantity, whereas that of the embryos in air only diminished gradually. At the change-over these effects were reversed; embryos formerly under nitrogen showed signs of revival, whilst those brought into nitrogen soon ceased to give off carbon dioxide.

The effect of cyanide on the respiration of excised embryos germinating in air was next examined. Two sets of 115 embryos each were set up on germination pads. One pad was saturated with M/200 potassium cyanide (pH 4.2 by HCl); and the other with M/200 potassium chloride similarly acidified. The germination chambers were connected with the gas-collection apparatus used in the leaf experiments. CO<sub>2</sub>-free air was passed through both chambers and oxygen and carbon dioxide both estimated. The results are

shown in Fig. 9. Oxygen absorption and carbon-dioxide emission are both depressed by the cyanide, as in the leaves. The effect on the carbon-dioxide emission is more striking than in leaves, since it actually exceeds the oxygen depression, causing the RQ to fall. This is consistent with the greater effect on  $\text{CO}_2$  emission of withholding oxygen.

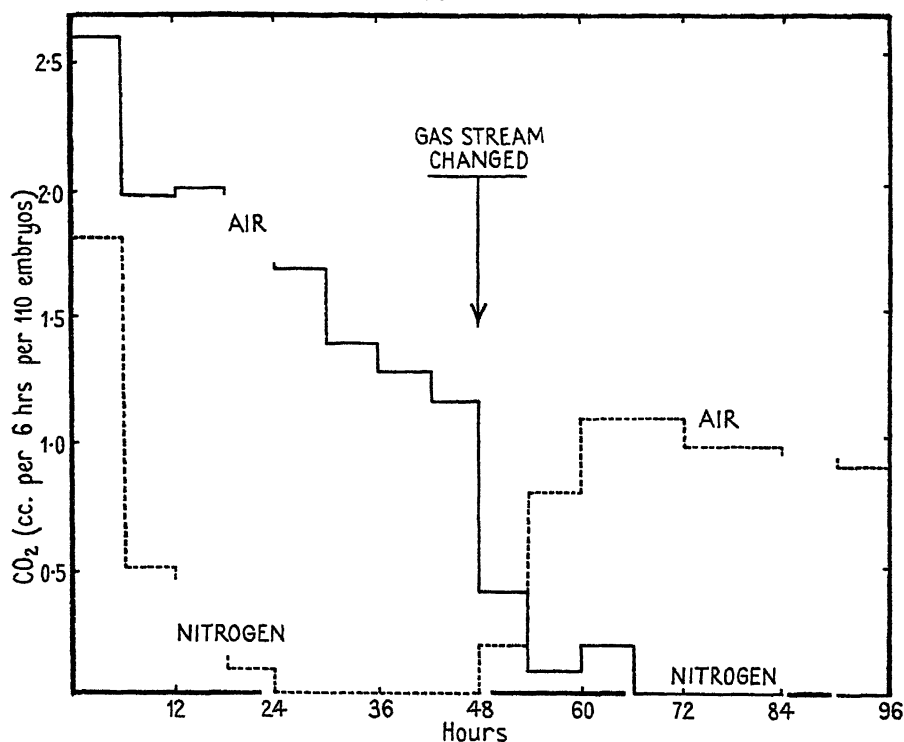


FIG. 8. Carbon-dioxide emission by 2 equal samples of excised embryos.

Although the suppression of  $\text{CO}_2$  output is not complete, as under nitrogen, the effect of the cyanide on germination is very striking. At the end of the experiment the unpoisoned embryos had sprouted satisfactorily, but those with cyanide not at all.

The germination of whole grains may be similarly inhibited by small doses of  $\text{H}_2\text{S}$ . Soaked grains were put into a germination chamber and the air passing over them first led through an Erlenmeyer flask containing an  $\text{H}_2\text{S}$  solution. The  $\text{H}_2\text{S}$  vapour carried over, completely inhibited germination.

#### DISCUSSION

It is clear from the foregoing experiments that the respiration of barley embryos and leaves is retarded by small doses of  $\text{HCN}$ . The oxygen consumption of leaves was reduced to about 36 per cent. by  $\text{M}/500$  cyanide; and the effect could be reversed by replacing the cyanide supply with water.

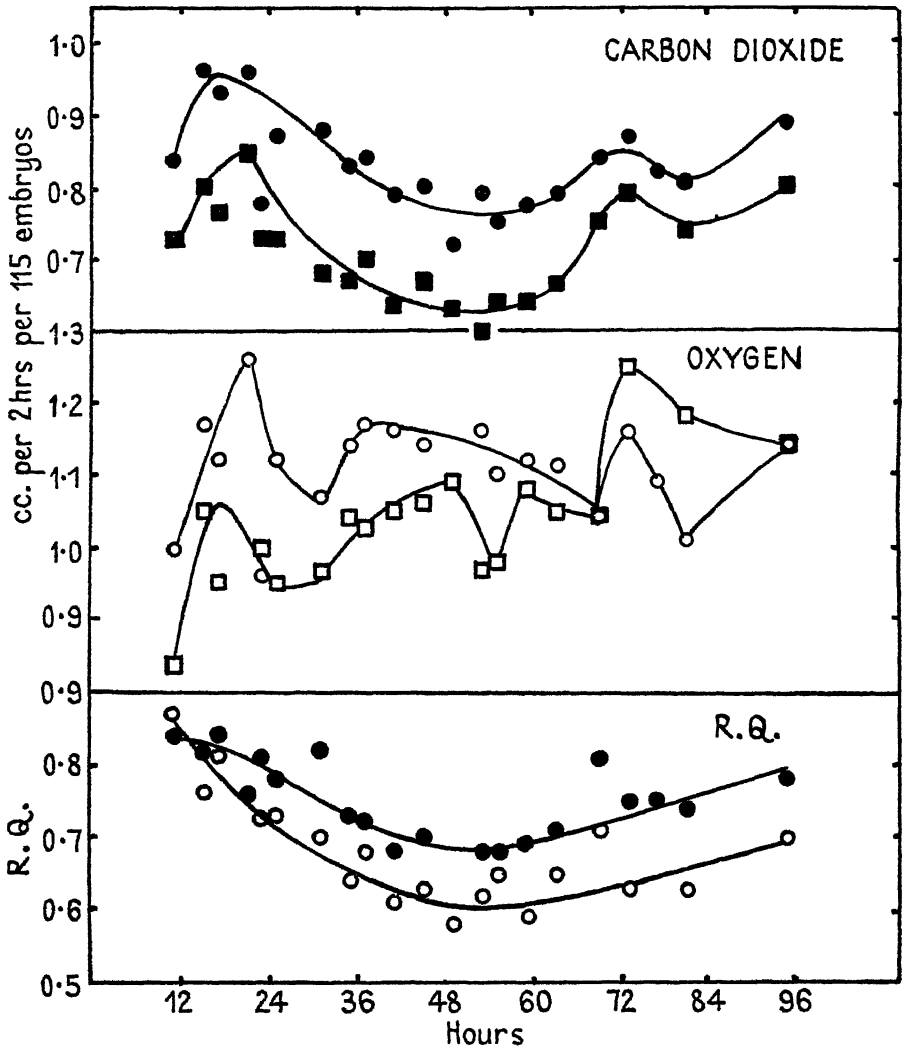


FIG. 9. Respiration of 115 excised embryos germinating in air. *Top* ●—● Carbon-dioxide emission with M/200 KCl. ■—■ Carbon-dioxide emission with M/200 KCN. *Middle* ○—○ Oxygen absorption with M/200 KCl. □—□ Oxygen absorption with M/200 KCN. *Bottom* ●—● RQ with M/200 KCl. ○—○ RQ with M/200 KCN.

With M/50 cyanide, the inhibition of oxygen uptake was rapid, complete, and not reversible by such simple means. Brief experiments with intermediate concentrations also showed depressions of oxygen intake.

In these experiments the carbon-dioxide emission was reduced less than the oxygen absorption, suggesting that the principal action of the cyanide was upon oxidation and that anaerobiosis was less affected. This was confirmed by the experiments under nitrogen, since during the first 24 hours they

showed no measurable depression of the  $\text{CO}_2$  output by cyanide such as is evident in air. It seems probable that the actual rate of sugar-breakdown (glycolysis) is also unaffected by cyanide. We have been able to show that yeast in the presence of M/500 cyanide gives a yield of  $\text{CO}_2$  from a given dose of sugar which corresponds closely with the fermentation equation (James and James 1936). The unaltered rate of  $\text{CO}_2$  production in these experiments, therefore, probably implies an unaltered rate of glycolysis. These results are in general agreement with those of Thomas (1937) for other species.

The depression of  $\text{CO}_2$  production which occurs later is at a stage when proteins have largely taken the place of the vanished carbohydrates as respirable material (see McKee, 1937). Proteolysis in barley leaves appears to be particularly sensitive to oxygen shortage. If oxygen is withheld carbon-dioxide emission is reduced at this stage, and application of cyanide under nitrogen causes still further reduction. The latter might still be due to the inhibition of an anaerobic oxidation involving water, perhaps, as oxygen supply. The slower  $\text{CO}_2$  output does not in itself prove a reduced rate of protein breakdown, but associated with these reduced respiration rates we have noticed a very marked postponement of yellowing.

After prolonged starvation the leaf-cells die, and their contents escape into the intercellular spaces, giving the leaf a translucent appearance. When this happens under pure nitrogen or with strong doses of cyanide in air, the leaves are often still fully green and give off a sweet, hay-like smell. In air, the leaves become completely yellow and may smell of ammonia. It seems clear that the inhibition of oxidation has markedly protected the leaf proteins. Reduction of oxygen pressure also retards protein and chlorophyll breakdown in *Tropaeolum* (Michael, 1935).

The equality of the  $\text{CO}_2$  emission-rates of leaves under air and nitrogen in the early stages of starvation does not imply that oxygen is without effect on glycolysis. Since a given quantity of sugar probably yields less  $\text{CO}_2$  under nitrogen than by complete oxidation in air, there was probably a faster rate of glycolysis under nitrogen, and a well-marked Pasteur effect in the presence of oxygen. The position is strikingly different with embryos in which  $\text{CO}_2$  emission is rapidly suppressed by the absence of oxygen and much reduced by dilute cyanide. Since there is scarcely any anaerobic  $\text{CO}_2$  production or germination, there is unlikely to be a Pasteur effect. Barnell (quoted from Turner, 1937), by a different method, has a similar result for barley grains germinated whole. There is still room for some uncertainty because the end products of anaerobiosis in barley have never been accurately determined.

#### SUMMARY

1. The oxygen uptake of barley leaves is reversibly inhibited by M/500 HCN. The inhibition is not complete.
2. Increasing concentrations of HCN have more marked effects. At M/50 inhibition is complete and is not reversed by removal of the cyanide supply.



3. In leaves carbon-dioxide emission is reduced less than is oxygen absorption, and the RQ rises.

4. Withholding oxygen from leaves does not alter the rate of carbon-dioxide emission before the period of the 'respiration hump'. This probably implies a more rapid breakdown of carbohydrate under nitrogen than in air, i.e. a well-marked 'Pasteur effect'. M/500 cyanide did not affect the CO<sub>2</sub> output under nitrogen at the same period.

5. The absence of oxygen retards the evolution of CO<sub>2</sub> at a later stage, when proteins are the principal respiratory substrate. The addition of M/500 cyanide under nitrogen retards it still further. Lack of oxygen or presence of cyanide both delay or prevent yellowing and the evolution of ammonia. It is to be presumed that proteolysis is correspondingly prevented.

6. The germination of excised embryos is inhibited by M/200 HCN or by the absence of oxygen. Small doses of H<sub>2</sub>S inhibit the germination of soaked grains.

7. The embryonic output of CO<sub>2</sub> is brought to a standstill under nitrogen in 24 hours, and is also reduced by cyanide as in leaves; the RQ falls in spite of a measure of oxygen inhibition.

These results suggest that an oxidation mechanism taking part in barley respiration is sensitive to dilute cyanide. Glycolysis is probably not directly affected, but the breakdown of proteins under nitrogen is.

We wish to thank the Department of Scientific and Industrial Research for a grant in aid; and the Christopher Welch Trustees for meeting the expenses of apparatus.

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# The Interaction of Light Intensity and Nitrogen Supply in the Growth and Metabolism of Grasses and Clover (*Trifolium repens*)

## III. Analytical Methods for the Estimation of Some Nitrogen and Carbohydrate Fractions

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With six Figures in the Text

### INTRODUCTION

THE previous two papers of this series (Blackman, 1938, and Blackman and Templeman, 1938) were concerned with the effect of varying light intensity and nitrogen supply on the clover (*Trifolium repens*) content of a sward. In the first paper it was demonstrated that the addition of nitrogen depressed the clover. The magnitude of the depression depended upon both light intensity and the source of the nitrogen; for ammonium sulphate was more effective than calcium nitrate in full daylight but not at lower levels of light. The second paper described experiments in which *Agrostis tenuis*, *Festuca rubra*, and *T. repens* were grown separately under a range of light intensities and nitrogen supply. It was found that while a reduction of the light intensity from daylight to 0.4 daylight depressed the leaf production of each species, the effects of additional nitrogen were related to the species. When the grasses were grown in full daylight ammonium sulphate and calcium nitrate increased leaf production to the same extent, whereas at lower light levels they caused a diminution; moreover, the diminution was greater with calcium nitrate. In contrast with the grasses the growth of *T. repens*, though dependent upon the light intensity, was not affected by varying nitrogen supply.

As a result of these findings it seemed clear that no adequate interpretation of the results was possible without information as to changes in the protein and carbohydrate metabolism of the leaves. It was decided therefore to estimate the various nitrogen fractions and also the readily available carbo-

hydrates such as starch and the sugars. This paper gives some account of the methods employed.

### EXPERIMENTAL RESULTS

All the chemical analyses were carried out on oven-dried plant tissues. It was realized that the analysis of quickly dried material was not as satisfactory as that of fresh tissue. Nevertheless, the large number of samples collected in a short space of time, i.e. up to 207 samples per day, rendered drying imperative. Within an hour of being cut, the leaves were spread out in a thin layer on a metal tray and placed in a special steam-heated oven in which hot air was forced over the trays. With the temperature maintained at 95° C. approximately the leaves were almost completely dry within an hour, and frequently sooner. After further drying the material was weighed, ground in a Christy-Norris micro-mill, bottled, and stored in the laboratory.

As for some of the samples only a very small quantity of leaves was available for chemical analysis, and as at the outset the scope of the analytical programme could not be foreseen micro methods were employed for the determinations when there was a paucity of material. In the majority of samples, however, there was a considerable bulk of leaves, and in these cases in order to minimize sampling errors determinations were made on a macro scale.

#### *Estimation of total nitrogen.*

For the determination of total nitrogen the Kjeldahl-Gunning technique was employed. For estimations on a macro scale a 1 gm. sample was placed in a Kjeldahl flask and, since it was known that the samples contained considerable amounts of nitrate nitrogen, salicylic acid and sodium thiosulphate were added as recommended by Frear (1891). In the digestions metallic selenium replaced the usual copper sulphate since the time of digestion was thereby shortened (Ashton, 1936). For the micro determinations the Kjeldahl apparatus as described by Pregl (1930) was used. The digestion was carried out after the addition of potassium and copper sulphates, and in order to include nitrates the material was first treated by the reduced iron method of Pucher, Leavenworth, and Vickery (1930).

A comparison of the values obtained by the two methods showed that almost without exception the macro technique gave a higher figure for the total nitrogen content. The data in Fig. 1 indicate that the disparity between the two methods is independent of the material analysed since the values for samples of *A. tenuis*, *F. rubra*, and *T. repens* all fall approximately on the same regression line. In order, however, to test this more exactly a further statistical analysis of the data has been carried out. It was considered that the most accurate information would be obtained by fitting for each species regressions of the *difference* between the two estimates on the nitrogen content as determined by the macro method. These ordinates were chosen since

there was evidence that the estimate obtained on the macro scale was more trustworthy in view of the higher values given, the closer agreement between duplicate analyses, and the likelihood of a smaller sampling error.

The straight-line regressions for the three species are shown in the top half

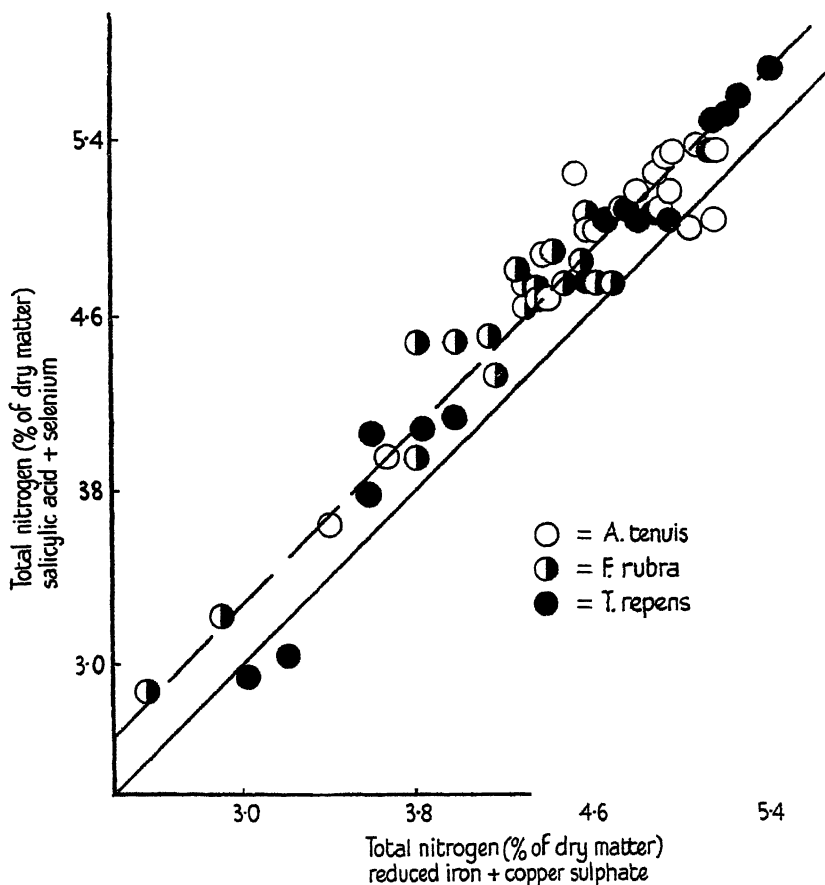


FIG. 1. Comparative values for Kjeldahl estimates of total nitrogen by two methods, (i) digestion with sodium thiosulphate salicylic acid and selenium (macro scale), (ii) digestion (micro scale) with potassium and copper sulphates after conversion with reduced iron of nitrate nitrogen to ammonia. The expected agreement is shown as a continuous line, the regression of closest fit to the data as a broken line. The plants are *Agrostis tenuis*, *Festuca rubra*, and *Trifolium repens*.

of Table I. On the basis of Fisher's notation (1925) the regressions have been expressed as

$$y = \bar{y} + b(x - \bar{x})$$

where  $y$  and  $\bar{y}$  are the individual and mean differences in nitrogen content as estimated by the micro and macro methods,  $b$  = the regression coefficient,

and  $x$  and  $\bar{x}$  are the individual and mean nitrogen contents as estimated by the macro method.

TABLE I

*Regressions of Difference in Total Nitrogen Content as estimated by Micro and Macro Methods on Estimate by Macro Method*

$$\textit{Agrostis tenuis} \quad y = -0.2833 - 0.0577(x - 4.9839)$$

$$\textit{Festuca rubra} \quad y = -0.3339 - 0.0065(x - 4.4481)$$

$$\textit{Trifolium repens} \quad y = -0.2050 - 0.0894(x - 4.7356)$$

*Analysis of Variance*

	Degrees of free- dom.	Sum of squares.	Mean square.	Z found.	Z test	
					P=0.05	P=0.01
Mean difference of nitrogen content for all species	1	4.0562	4.0562	2.5055	—	1.0116
Interaction for species	2	0.1519	0.0760	0.5138	0.5738	—
Mean regression of difference on macro nitrogen for all species	1	0.1465	0.1465	0.8454	0.7141	—
Interaction for species	2	0.0029	0.0015	—	—	—
Error	48	1.3062	0.0272	—	—	—

It is seen that the regression lines for the three species are in close agreement. In fact it is clear from the analysis of variance (Table I) that the three regressions are not significantly different. While the average discrepancy between the two methods is highly significant the lower value given by the micro method is not dependent upon the nature of the material analysed as the interaction for species is not significant. Similarly, the magnitude of the difference is dependent on the nitrogen content of the sample since the mean regression on 'macro' nitrogen content is significant. This relationship is not, however, dependent on the species as the regression interaction is not significant.

*Estimation of protein nitrogen.*

For the estimation of protein nitrogen two methods were employed. When a detailed analysis of the non-protein fractions was not carried out the protein was precipitated from a 1 gm. sample (boiled for 5 minutes with 100 c.c. water) with Stütze's copper hydroxide-glycerol reagent and the nitrogen in the precipitate estimated by the macro procedure as previously described.

With some samples, however, the various non-protein nitrogen fractions were determined separately, and in these cases trichloroacetic acid was employed to precipitate the protein. In this way a more or less colourless filtrate containing the non-protein nitrogen was obtained which facilitated the use of colorimetric methods for the subsequent estimations of the different non-protein fractions. Thus the protein nitrogen was not determined directly but obtained by subtracting the non-protein nitrogen from the total nitrogen

when both estimates were carried out by the micro method of Pregl. The non-protein nitrogen was extracted as described by Richards and Templeman (1936), and any protein present in the solution was precipitated by adding 5 per cent. of 50 per cent. trichloroacetic acid.

TABLE II

*Regressions of Difference in Protein Nitrogen Content as estimated by Stütze Macro and Trichloroacetic Acid Micro Methods on Estimate by Stütze Macro Method*

$$\textit{Agrostis tenuis} \quad y = -0.0725 + 0.0917 (x - 4.0713)$$

$$\textit{Festuca rubra} \quad y = 0.0200 + 0.0264 (x - 3.4788)$$

$$\textit{Trifolium repens} \quad y = +0.1725 - 0.0258 (x - 3.7175)$$

<i>Analysis of Variance</i>					
	Degrees of freedom.	Sum of squares.	Mean square.	Z found.	Z test P = 0.01
Mean difference in protein estimate for all species	1	0.0016	0.0016	—	—
Interaction for species	2	0.2817	0.1409	1.3167	0.8970
Mean regression of difference in protein estimate on macro-estimate	1	0.00117	0.00117	—	—
Interaction for species	2	0.01215	0.00607	—	—
Error	18	0.18224	0.01012	—	—

The comparative values obtained by the Stütze macro nitrogen method and the trichloroacetic micro nitrogen method are given in Fig. 2. It is seen that although the macro method gives on an average a higher estimate ( $P = 0.01$ ) the differences are by no means consistent. As the micro technique had already been shown to give a lower value for the total nitrogen than the macro technique it was thought that a closer agreement might be obtained if the non-protein nitrogen figure, as determined by the micro method, was subtracted from the total nitrogen estimate as determined by the macro method. From the data in Fig. 3 it is clear that this adjustment brings about a better agreement between the two methods.

In order to test the agreement more accurately the data have been treated statistically in a similar way to that described for the total nitrogen results (see p.120). The straight-line regressions of the difference in protein nitrogen content as estimated by the trichloroacetic acid and Stütze methods on the protein nitrogen as determined by the Stütze method are shown in Table II, together with the analysis of variance. The data show that whereas for the grasses the two methods give a reasonably good agreement, in the case of the clover the trichloroacetic acid mean value is higher. That this difference is highly significant is shown by the analysis of variance since 'Z' for the interaction for species is less than 0.01. As neither the mean regression nor the regression interaction are significant the agreement or disagreement between the methods is independent of the protein nitrogen level.

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All these estimations were carried out on the micro scale. After precipitation of the protein by trichloroacetic acid aliquots of the filtrate were taken

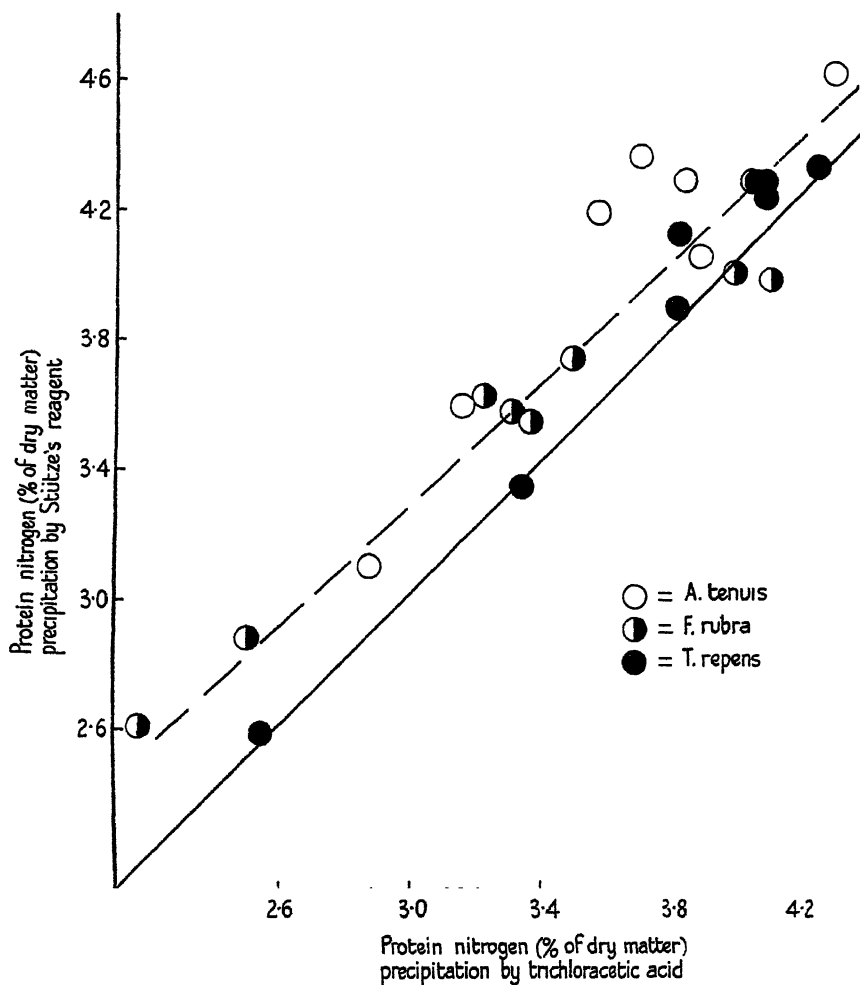


FIG. 2. Comparative values for Kjeldahl estimates of protein nitrogen by two methods, (i) digestion of precipitate obtained with Stütze's reagent, using selenium as catalyst, (ii) precipitation of protein with trichloroacetic acid and estimation of total nitrogen and non-protein nitrogen by reduced iron copper method; protein nitrogen by difference. The expected agreement is shown as a continuous line, the regression of closest fit to the data as a broken line. Plants as in Fig. 1.

for the various determinations. Amino nitrogen was determined by the Sørensen formol titration method as modified by Brown (1923). For the amide fraction the solution was hydrolysed with sulphuric acid, neutralized

with soda as suggested by Vickery and Pucher (1931), and the resulting ammonia estimated by Wolff's (1928) method. Ammonia nitrogen was determined in a similar way after the solution had been neutralized with magnesia cream.

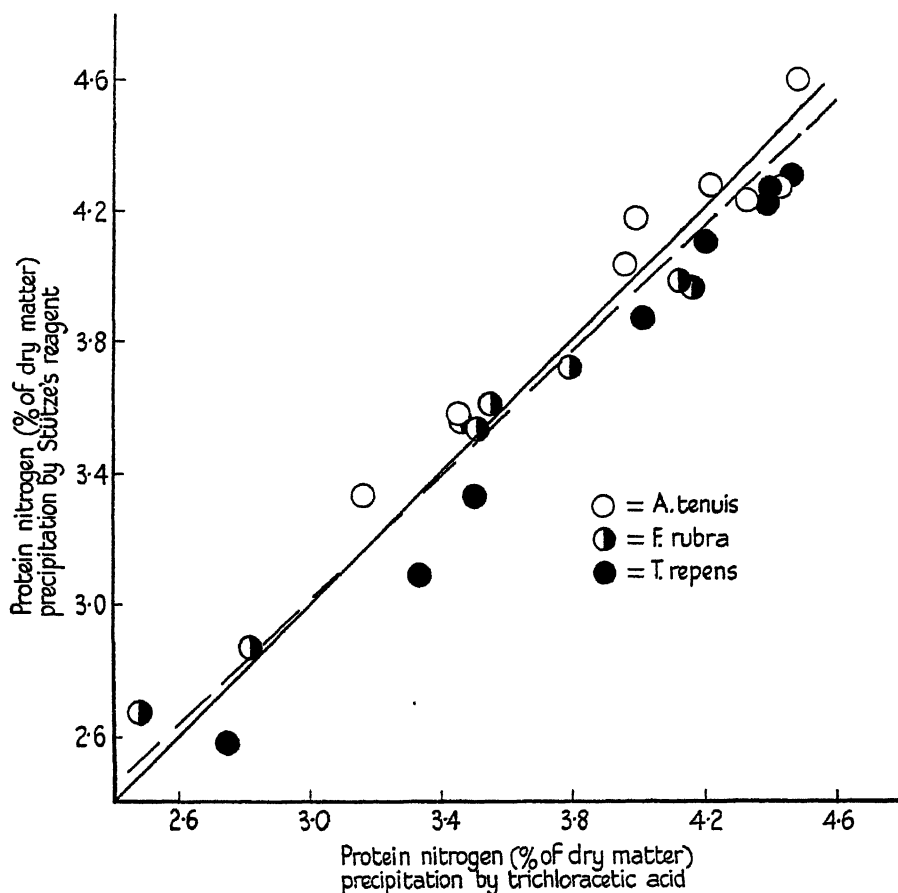


FIG. 3. Comparative values for Kjeldahl estimates of protein nitrogen by two methods, (i) digestion of precipitate obtained with Stütze's reagent, using selenium as catalyst, (ii) precipitation of protein with trichloroacetic acid and estimation of total nitrogen by the selenium-salicyclic acid method, and of non-protein nitrogen with reduced iron copper; protein nitrogen by difference. The expected agreement is shown as a continuous line, the regression of closest fit to the data as a broken line. Plants as in Figs. 1 and 2.

#### Estimation of nitrate nitrogen.

Estimations of the nitrate were carried out in two different ways. On the micro scale, the alkaline residues from the amide estimation were treated in the cold with Devarda's alloy, and after the reduction was complete the ammonia estimated by Wolf's method.



During the course of the analyses it became clear that of the non-protein fractions the nitrate nitrogen was by far the most important physiologically and also present in the largest amounts. It was decided, therefore, that the extra labour and time involved did not warrant for every sample the complete partition of the non-protein nitrogen fractions.

For the estimation of the nitrates the direct colorimetric method of Ashton (1935) was first employed. In this procedure equal quantities of dried material and magnesium oxide are boiled in a small quantity of water for five minutes to remove any ammonia nitrogen present. After the solution has been allowed to cool it is cleared with potash alum and lead subacetate and finally, before filtering, silver sulphate is added to precipitate any chloride present. The filtrate is made up to a standard volume and a small aliquot placed in a porcelain basin and evaporated to dryness on a water-bath. In order to remove the last trace of colour, 0.1 c.c. of 100-volume hydrogen peroxide is added to the solution. After the evaporation is complete, phenoldisulphonic acid is run in, and subsequently the depth of the characteristic yellow colour developed in alkaline solution estimated in a colorimeter.

After a number of determinations had been made by this method it was clear that the estimates obtained were much lower than those given by the Devada's alloy-Wolff method. It seemed probable that there was a loss of nitrate as a result of clearing with alumina cream and lead subacetate. Attempts were therefore made to obtain a reasonably colourless solution without clearing and the final procedure adopted was as follows. One gm. of the material and 1 gm. of magnesium oxide are boiled with 75 to 100 c.c. of water for five minutes. After cooling, 2–3 c.c. of saturated silver sulphate solution are added and the solution is allowed to stand for ten to fifteen minutes. If at the end of this time the solution has not turned reddish-brown as a result of the formation of colloidal silver oxide the procedure is repeated, but no more silver sulphate is added once the red tint appears. The solution is then filtered, the filtrate made up to 250 c.c. and a sample of 10 c.c. pipetted into an evaporating basin. After the basin has been placed on a water-bath, 0.1 c.c. of 100-volume hydrogen peroxide is immediately added and the solution evaporated to dryness. The residue is mixed with 1 c.c. phenoldisulphonic acid and stirred with a small glass rod; approximately 20 c.c. of water is then run in, the liquid stirred again until there is no residue left, and finally 10 c.c. of 1 in 3 strong ammonia solution added. The whole is then washed into a 50 c.c. flask and the solution made up to the mark. The yellow colour developed is matched against standards of known nitrate content in a colorimeter.

With the materials used in this investigation the final solutions obtained before the addition of the ammonia were reasonably colourless. Provided that excess silver sulphate was not added there was but a very faint yellow colour, amounting at the most to an error of 0.01 per cent. of nitrate nitrogen on a dry matter basis. If silver sulphate was in excess the filtrate contained considerable amounts of colloidal silver oxide and then the final colour was

both somewhat deeper—corresponding to 0.025 per cent. of nitrate nitrogen—and different in tint from that of the standard. The interference effects of such colours can, however, be largely eliminated if a two-stage colorimeter is employed.

Using 1 gm. of dried material and the procedure outlined above, it was found possible to estimate satisfactorily the content of samples containing 0.025 per cent. of nitrate nitrogen. With a Klett colorimeter diffuse daylight was used as the source of light but, when a Hellige Dubosc instrument was employed matching was easiest with electric light and a blue filter. If the material contained more than 0.5 per cent. of nitrate nitrogen then the solution was diluted, otherwise the depth of the yellow colour made matching difficult.

Using the modified technique without clearing agents, determinations of the nitrate content were repeated and found to give higher values than those obtained when the solution was cleared. The magnitude of these differences is shown in Fig. 4. Assuming that the estimates obtained without clearing are valid determinations, then on the basis of the fitted straight line regression clearing removes on the average some 40 per cent. of the nitrate present in the solution. Further experiments showed that clearing with either alumina cream or lead subacetate alone resulted in the removal of nitrates from the solution.

Finally, a comparison was made between the colorimetric technique and the micro method, namely, the reduction of the nitrate by Devarda's alloy and the estimation of the resulting ammonia by Wolff's method. The excellent agreement between the two methods is seen in Fig. 5.

#### *Estimation of water-soluble carbohydrates.*

For the determination of water-soluble carbohydrates the copper reduction methods as modified by van der Plank (1936) and Archbold (1938) were employed. The sugars were extracted by shaking mechanically for one hour 1 gm. of material and 30 c.c. of cold water. The mixture was then filtered, the residue washed with approximately 60 c.c. of water, and the filtrate made up to 100 c.c. This constituted the stock solution and was used without clearing for all determinations.

For the 'total sugar' estimations aliquots of the solution were placed in boiling tubes, acidified to 0.2 N with sulphuric acid and the tubes placed in boiling water for ten minutes. After cooling the acid was neutralized with caustic soda and the reducing sugars estimated.

In order to test whether the carbohydrates in the extract consisted largely of sucrose and reducing sugars, a comparison was made between inversion with acid and inversion with invertase. Since the figures obtained for inversion with invertase were 85–93 per cent. of the acid values it was concluded that the bulk of the hydrolysable material was sucrose. Some of the solutions were tested for fructosan by the modified Koltoff method of van der Plank (1936) and Archbold (1938), and were found to contain no appreciable amount of this substance.

*Estimation of reducing sugars.*

In the initial determinations of the reducing sugars, the sugars were extracted from the material with hot 95 per cent. alcohol in a Soxhlet apparatus. To the alcoholic extract water was added and the alcohol removed by distillation in a partial vacuum at a temperature not exceeding 40° C. The remain-

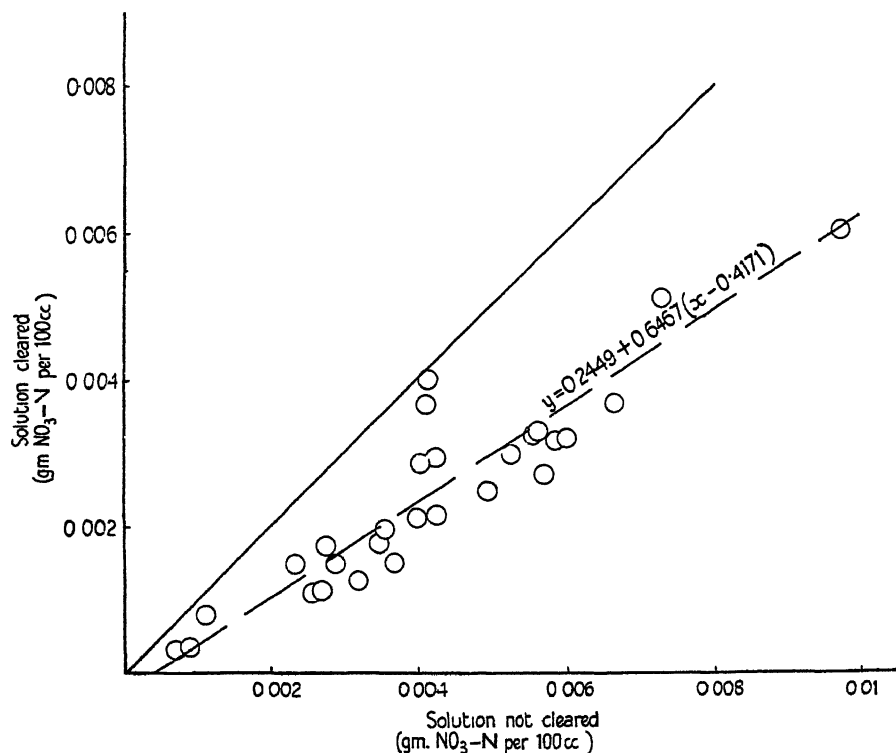


FIG. 4. Effect of clearing with alumina cream and lead subacetate on the nitrate nitrogen content of plant water extracts: nitrate estimated colorimetrically with phenoldisulphonic acid. The regression of closest fit to the data is shown as a broken line, the relationship, if there was no loss of nitrate by clearing, as a continuous line.

ing liquid in the distillation flask was then made up to a known volume and the reducing sugars estimated on aliquot samples.

As the procedure outlined above was somewhat laborious, the feasibility of estimating the reducing sugars direct after extraction with water was investigated. Since the material had been dried at 95° C. it seemed highly probable that all the enzymes present had been inactivated. On this assumption there would be no conversion of the sucrose during extraction with water and the reducing sugars could be determined in the extract prior to the acid inversion of the sucrose.

To test for complete inactivation of the invertase the following procedure was adopted. Of material whose content of reducing sugars had been previously estimated by the hot alcohol extraction method, 1 gm. samples were shaken up with water and the filtrates made up to 100 c.c. On an aliquot

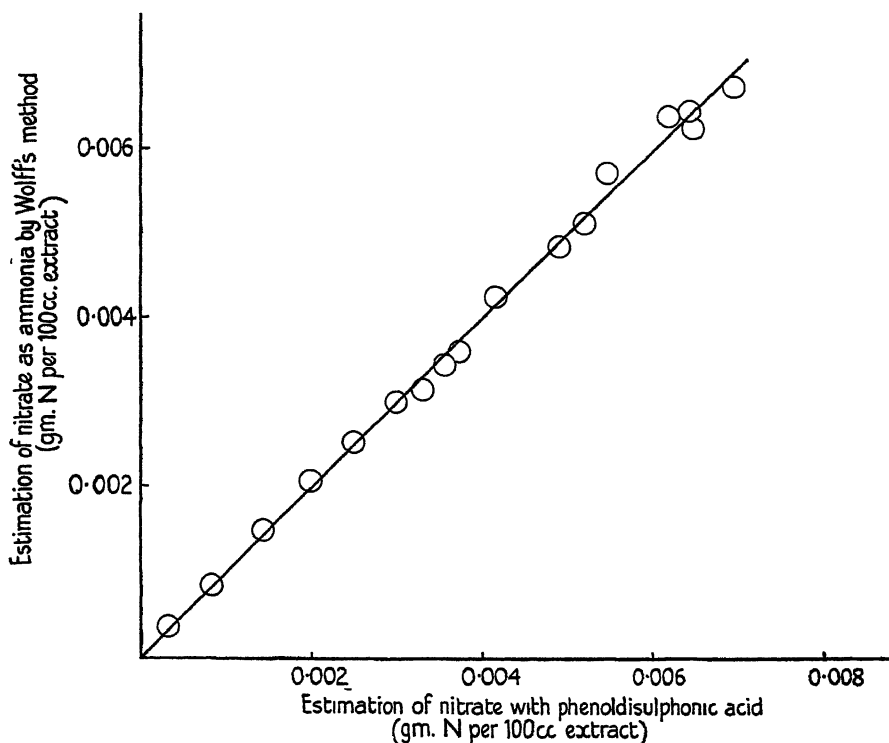


FIG. 5. Comparative values for estimates of nitrate nitrogen by two methods, (i) conversion of nitrate nitrogen to ammonia with Devada's alloy and estimation of ammonia by Wolff's method, (ii) direct colorimetric determination using phenoldisulphonic acid.

sample of each filtrate the reducing sugar content was again estimated. The remaining solution with the addition of a few c.c. of toluene was placed in bottles with ground-glass stoppers and incubated at 24° C. From time to time samples were withdrawn for further analyses.

The curves in Fig. 6 show quite clearly that despite the high drying temperature the samples still contained active invertase. At the end of three weeks all the sucrose present had been converted, since the amount of reducing sugars present in the solution was the same as that obtained from comparable extracts after inversion of the solution with 0.02 per cent. invertase (vide Table III). In consequence of this somewhat surprising result the estimation of reducing sugars in the water extract was abandoned.

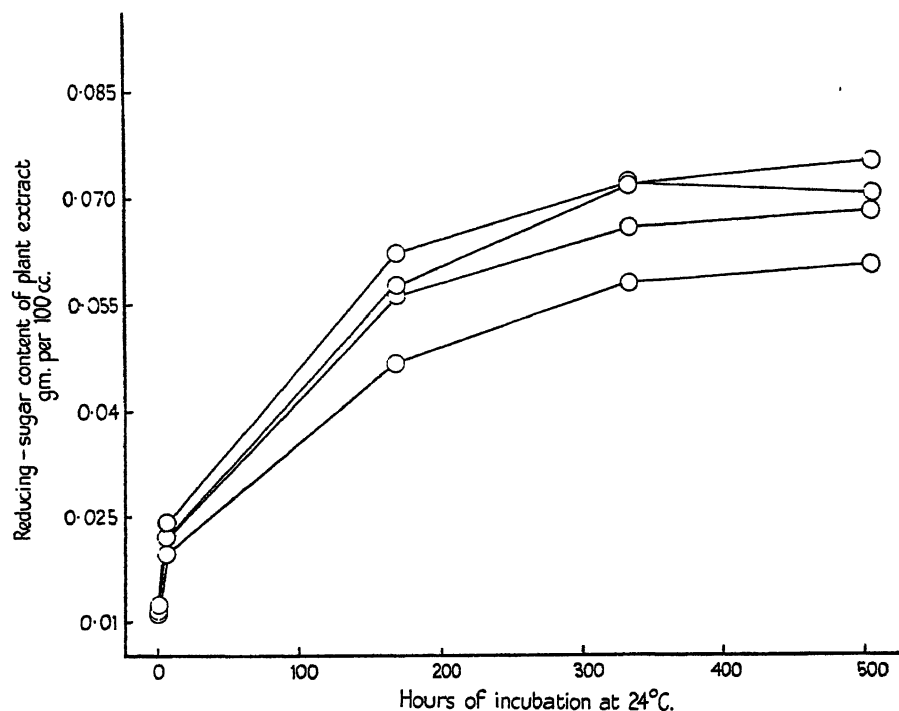


FIG. 6. Increase with time in the reducing-sugar content of water extracts as a result of enzymic inversion of sucrose; extracts from leaves previously dried at 95° C.

TABLE III

*Inversion of Sucrose in Water Extracts of Leaves dried at 95° C.*

Sample.	Initial content.	Final content after incubation for 504 hours.	Content of reducing sugars (gm. per 100 c.c. of extract).	
			Content after inversion (a) invertase.	(b) dilute acid.
1	0.0122	0.0750	0.0760	0.0830
2	0.0114	0.0682	0.0673	0.0723
3	0.0116	0.0704	0.0724	0.0740
4	0.0114	0.0605	0.0611	0.0650

*Estimation of starch.*

Examination of the samples under the microscope after they had been stained with dilute iodine showed that considerable quantities of starch were present in the leaves of clover grown in full daylight. At no light intensity could starch be detected in the leaves of the grasses, while in the majority of clover leaves grown under the lowest light intensities (0.43–0.37 of daylight) only traces of starch were found.

For the determination of starch after some modification the methods of Haines (1936) were adopted. The first tests were carried out on samples from which the sugars had been extracted with alcohol in a Soxhlet apparatus. It was found that when the residue was boiled in a reflux condenser with the alcohol hydrochloric acid mixture (100 c.c. 95 per cent. alcohol, 1 c.c. concentrated acid) for twelve minutes as recommended by Haines not all the starch was rendered soluble. As a result after the sample had been washed with alcohol to remove the hydrochloric acid and the starch subsequently extracted from the dried material with hot water (boiled for three minutes and held at 100° C. for a further twenty minutes) the final residue when tested with iodine still contained starch in some of the cells. If, however, the material was boiled with the alcohol acid mixture for an hour instead of twelve minutes and subsequently extracted with hot water for an hour then the final residue was almost starch free.

Since the initial extraction of the sugars in a Soxhlet apparatus was laborious, and since a large number of starch determinations was necessary, the quicker method of water extraction (see p. 127) was adopted. It was realized that during the time (two hours) the material was in contact with the water some of the starch might have gone into solution, but tests with iodine on the final solution never gave a blue coloration.

Such negative tests are, however, not conclusive since some of the starch may have gone into solution after conversion with enzymes if the starch-converting enzymes had not been completely inactivated by oven drying. That complete inactivation had not taken place was demonstrated by storing samples with water and a few c.c. of toluene in an incubator at 38° C. and from time to time testing with iodine the material for starch. By the end of two to three weeks most of the starch had disappeared. On the other hand, when the tests were repeated and the samples stored at room temperature there was still considerable starch in the tissue at the end of five weeks. If, therefore, during the extraction with cold water some of the starch was hydrolysed by enzyme action, the amount converted in a period of two hours must have been negligible.

In the final extract the starch was converted to maltose with Haine's  $\beta$ -amylase preparation and the reducing power of the solution estimated as for the water-soluble carbohydrates. No attempt was made to isolate the starch in the clover leaves and use such material for the construction of a calibration curve; for this purpose pure starch was employed instead.

## DISCUSSION

The results obtained in this investigation illustrate some of the difficulties that arise in the chemical analysis of plant material. Whereas in the estimation of total nitrogen in leaves the discrepancy between the two methods was independent of the plant used, yet in the estimate of the protein nitrogen by the two methods employed the agreement was dependent on the species.

In the case of the total nitrogen, the 6.5 per cent. higher mean value over the micro method obtained by digesting the material by the macro method is most probably due either to the greater efficiency of selenium compared with copper as a catalyst, or to the relative effectiveness of salicylic acid and the reduced iron methods for the conversion of the nitrate to ammonia nitrogen. No attempt has been made to investigate these possibilities since the primary object of the work was to obtain reliable and comparable methods suitable for the routine analysis of a large number of samples.

The interdependence of the source of the material and the methods of estimating protein nitrogen is not surprising from the biological point of view. Since the division between proteins and the amino acids brought about by either Stütze's reagent or trichloroacetic acid cannot at present be considered exact, it would not be unexpected for the trichloroacetic acid to precipitate amino acids which remained unprecipitated by Stütze's reagent. The higher value for protein given, therefore, by trichloroacetic acid in the case of the clover leaves suggests that these differed in composition from those of the grasses. That this is so is indicated by analytical data. Comparable analyses reveal that the clover plants contain a higher percentage of non-protein nitrogen, due to a greater content of both amino and amide nitrogen and somewhat less nitrate nitrogen.

Such effects cannot be ascribed to the difference in the method of estimating the total nitrogen. If the value for the non-protein nitrogen is obtained by subtracting the total nitrogen from the protein nitrogen (Stütze), both determined by the macro method, then the mean values obtained for *A. tenuis*, *F. rubra*, and *T. repens* are 0.705, 0.7025, and 1.213, while the comparable averages for the non-protein nitrogen determined directly by the micro nitrogen method, after removal of the protein by trichloroacetic acid, are 0.775, 0.691, and 1.024. If the composition of the plant material was not involved, then, on the basis of the comparison of the nitrogen methods, the non-protein nitrogen determined by difference should be with each species approximately 6–7 per cent. greater. This clearly is not so; the differences in the cases of the two grasses is not significant, but for clover the 15.6 per cent. lower value for the trichloroacetic determination is significant ( $P = 0.01$ ).

The presence of active enzymes in the dried leaves gives some measure of the high efficiency of the oven in rapidity of drying and suggests that little change in composition has taken place during drying. The failure of a final temperature of 95° C. to inactivate at least some of the enzymes must be associated with this rapid desiccation. If a large proportion of the water is lost within a short space of time then no doubt during this period the material is at a temperature considerably below that of the oven. Subsequently the temperature of the tissues will rise sharply, but the enzymes in the material by now much desiccated presumably are less thermolabile. As has been demonstrated, the presence of enzymes adds to the complexity of chemical analysis. Either they must be destroyed by boiling the samples with water (Stütze

protein or colorimetric nitrate nitrogen methods), or if this heat treatment is impossible the material must be analysed as soon as the extraction is complete. The storage of extracts for any length of time prior to analysis may introduce considerable errors.

#### SUMMARY

During a study of chemical methods suitable for the routine analysis of a large number of samples the following results have been obtained:

Using the Kjeldahl-Gunning technique for the estimation of total nitrogen it has been found that digestion with sodium thiosulphate, salicylic acid, and metallic selenium on a macro scale gives values which are on average 6.5 per cent. higher than those obtained on a micro scale in which the samples are digested with potassium and copper sulphates after any nitrate nitrogen has been converted to ammonia by the reduced iron method. Statistical treatment of the analytical data for leaves of *Agrostis tenuis*, *Festuca rubra*, and *Trifolium repens* shows that the size of the discrepancy between the two methods is dependent upon the nitrogen content of the sample but not on the kind of leaf.

The protein nitrogen was determined (i) directly by digesting the precipitate obtained with Stütze's reagent, and (ii) indirectly by determining the non-protein nitrogen after the removal of the protein with trichloroacetic acid and subtracting this estimate from the total nitrogen figure. When for the total nitrogen and Stütze protein values selenium was used as a catalyst and for the non-protein nitrogen copper sulphate, then the direct and indirect methods gave good agreement in the case of the two grasses; but with leaves of the clover the trichloroacetic acid estimates were significantly higher.

A method for the rapid estimation of nitrate nitrogen colorimetrically is described. It was found that a preliminary clearing of the solution with alumina cream and lead subacetate removed variable amounts of nitrates and thereby introduced large errors.

Although the leaves were dried in an oven with an air temperature of approximately 95° C. the enzymes were not completely inactivated. In material so dried the presence of enzyme systems capable of converting both sucrose and starch was demonstrated. In the chemical analyses, particularly of the carbohydrate fractions, this factor may on occasion give rise to considerable errors.

The authors are indebted to Imperial Chemical Industries, Ltd., for permission to publish some of these results. They also wish to thank Dr. H. K. Archbold for advice concerning the carbohydrate analyses and Dr. M. S. Bartlett for help in the statistical treatment of the data.

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# The Mycorrhizal Relations of Larch

## I. A Study of *Boletus elegans* Schum. in Pure Culture

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With sixteen Figures in the Text

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## I. INTRODUCTION

THE regular occurrence of many soil Hymenomycetes in the vicinity of certain species of trees is well known to field mycologists. It is now realized that in many cases this is the outcome of a mycorrhizal relationship between tree and fungus. By far the most constant of these associations recorded for *Boletus* is that between *B. elegans* and the larch, a fact commented upon many times in the last 100 years (cf. Melin, 1922, for detailed bibliography); in this particular instance the mycorrhizal basis has been established. It seemed likely that an investigation of such a specialized relationship would throw light both on fungal physiology and the role of mycorrhiza in trees. With this in view Melin (1922) published a preliminary investigation into the physiology of *B. elegans*; a study which was never completed, however, as larch seedlings proved difficult subjects for pure culture work. The present investigation set out to enlarge Melin's observations and to discover, if possible, the cause of this specialized relationship by means of a morphological and physiological study of *B. elegans* in pure culture.

## II. ISOLATION OF THE FUNGUS

Tissue culture is the only reliable method for the isolation of fungi such as *B. elegans* the spores of which have rarely, if ever, been germinated. Repeated isolations have been made from young sporophores, which were either flamed or washed in 0.1 per cent. mercuric chloride for one minute and repeatedly washed in sterilized distilled water. After sterilization pieces of tissue were cut from the pileus above the stipe and were transferred to the following nutrient agar medium (Melin, 1922):

Glucose	.	.	.	.	.	.	20.0 gm.
MgSO <sub>4</sub> ·7H <sub>2</sub> O	.	.	.	.	.	.	0.1 gm.
NH <sub>4</sub> Cl	.	.	.	.	.	.	0.5 gm.
KH <sub>2</sub> PO <sub>4</sub>	.	.	.	.	.	.	1.0 gm.
Agar-agar	.	.	.	.	.	.	20.0 gm.
Distilled water	.	.	.	.	.	.	1,000 c.c.

Stock cultures were at first grown alternately on glucose and beerwort agar, but later mainly beerwort agar was used, with an occasional transference to glucose agar, 2 per cent. malt or potato dextrose agar; a continuous diet of any one medium leads to a lowering of the growth rate. Melin's observations (1925) of the necessity of subculturing at least once a month were confirmed; there is then no loss of vigour even after three or four years.

## III. MYCELIAL CHARACTERS

The main points in the description of the Swedish strain of *B. elegans* given by Melin (1922) are as follows: The cultures are white at first with rust-yellow patches appearing in old colonies and a dark brown pigment diffusing out under certain conditions. The hyphae are septate, 2–3  $\mu$  wide and frequently branched; the branches emerge singly at an angle of 45° immediately below a transverse wall to give the so-called 'simple branching' (Figs. 1 and 2). Hyphal strands occur in old cultures, hyphal fusions are also found, but clamp connexions do not occur. The aerial hyphae are covered with granular secretions giving a papillated appearance; no true spores or conidia are formed.

Examination of the English strain of *B. elegans* isolated by the writer has revealed a few points in the macroscopic appearance of the cultures to be added to the description of the Swedish strain given above. In the English strain the rust-yellow patches are a feature of glucose media only, brown patches being developed on oat or beerwort media. In glucose agar cultures a bright yellow substance diffuses out under certain conditions not determined; this yellow substance seems to be distinct from the brown substance mentioned by Melin which normally occurs in all nutrient agar cultures. Drops of a brown, oily fluid appear on the surface of the cultures when growth is strong, and occasionally brown crystals form on the surface of the agar beyond the margin.

Microscopic examination has shown that the two strains differ in a number

of features which are of importance in a comparison of this species of *Boletus* with others; these are considered in detail below.

### Branching.

Melin (1923) describes two types of branching in the genus, simple branching as described above and 'paarige' branching in which a pair of branches is given off immediately below a transverse wall. According to Melin, while many species exhibit both types, *B. elegans* shows only simple branching. In the English strain of the fungus, however, 'paarige' branching occurs frequently on both substrate and air hyphae (Figs. 1 and 2). Nor after four years' growth in culture is there any evidence that 'paarige' branching is becoming less frequent in occurrence or that it is confined only to the vigorous cultures as Melin found was the case for *Mycelium radices sylvestris*  $\alpha$  and *Boletus* spp.

Structures resembling clamp connexions have been observed only twice in this investigation, it is therefore probable that the English and Swedish strains are similar in this respect. No reliance can be placed on the reported occurrence of clamp connexions in basidiospore cultures of *B. elegans* by Hammerlund (1923), since no figures are given and his soil media were inadequately sterilized. Examination of the mycelium below the sporophores has also failed to establish the occurrence of such connexions in natural soil conditions.

From Melin's work it is possible to classify the Boleti into two groups as follows:

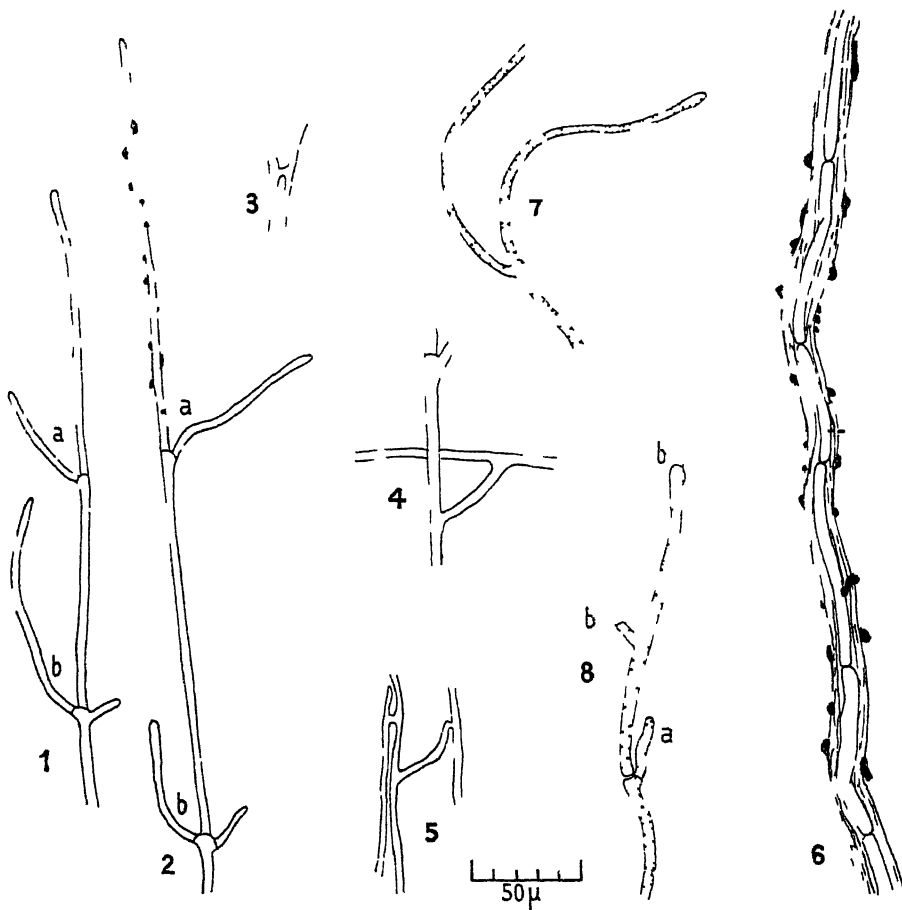
1. Mycelium with frequent 'paarige' branching and numerous clamp connexions, e.g. *B. luteus* and *B. variegatus*.
2. Mycelium with infrequent 'paarige' branching and few if any clamp connexions, e.g. *B. granulatus* and *B. badius*.

While the Swedish strain of *B. elegans* is to be placed in group 2, the English strain exhibits features of both groups, supporting the view that this species does not differ seriously from other members of the genus already described.

### Hyphal fusions and hyphal strands.

In contrast to the Swedish strain of *B. elegans* the English strain frequently develops bridging hyphae, especially among the hyphae on the surface of the substrate (Figs. 3, 4, and 5).

Hyphal strands 20  $\mu$  in diameter are commonly found on beerwort or oat agar cultures after three weeks' growth. Occasionally the English cultures have produced hyphal strands 45–6  $\mu$  thick, which compare favourably with the strands figured by Melin (1922) from cultures in symbiosis with seedlings of larch; these were 40  $\mu$  in diameter. Many of the strands examined consist of a central axis of wide hyphae containing glycogen and surrounded by a sheath of narrow hyphae (Fig. 6). These strands show an organization similar to that found in hyphal strands of other Hymenomycetes, e.g. mushroom (Hein, 1930), though the degree of differentiation is less, only two types of hyphal element being involved.



FIGS. 1-8. Figs. 1 and 2. Aerial hyphae from 4-day-old glucose gelatin culture: *a*, simple branching; *b*, 'paarige' branching (drawn from living material). Figs. 3, 4, and 5. Substrate hyphae showing hyphal fusions from a 6-day-old glucose gelatin culture. Fig. 6. Hyphal strand from a 2-months-old beerwort agar culture. Fig. 7. Part of branched 'brown' hypha from 7-weeks-old oat agar culture. Fig. 8. Immature 'brown' hypha from 2-months-old beerwort agar culture: *a*, hyaline branch beginning to change colour; *b*, unchanged parts of original hyaline hypha. All cultures of a strain 1½ years old.

### *Brown aerial hyphae.*

Examination of the brown patches which appear after ten days' growth on sugar or starch media reveals the presence of numerous disconnected hyphal threads with yellow-brown walls (Fig. 7). These hyphae are mostly unbranched and usually terminate abruptly with blunt end-walls forming relatively short threads; occasionally hyphal bridges are observed between two such hyphae. These brown threads always occur on the outer surface of the mycelial mat; varying degrees in the intensity of the yellow-brown colour are found, depending on the age of the culture, the older the culture the darker the colour.

The brown hyphae appear to arise through a modification of the walls of segments of normal, hyaline, aerial hyphae. Occasionally it is possible to find a brown hypha continued at both ends into a normal hyaline hypha (Fig. 8), but in most cases the unmodified portions of the hyphae have shrivelled, leaving the typical isolated brown fragments. The increasing depth of colour with age suggests the gradual deposition of the yellow-brown substance in the walls. Melin (1923) describes and figures hyphae in *Mycelium radialis abietis* which appear to be of a similar nature and are stated to be rich in glycogen; however, there were only traces of glycogen in the brown hyphae of *B. elegans*. Hyphae similar to those in *B. elegans* have been observed in large numbers in cultures of *B. bovinus*; it is probable that they are a general feature of *Boletus* spp. in culture, waste products of metabolism being deposited in this manner.

### Secretions.

A very constant feature of the mycelium of *B. elegans*, both in culture and in the soil, is the presence to a greater or less degree of masses of granules on the walls of the hyphae giving them a papillated appearance. Melin noted that the granules vary in their degree of attachment to the hyphae, the small ones not being removed by washing with water or alcohol, the larger easily separating. In the present investigation it was found that the small granules stain deeply with aqueous methylene blue; the larger stain less easily, are partly oily in nature and brown in colour, giving rise with the brown hyphae to the rust-yellow or brown patches on old cultures. These large granules are probably older stages of the small granules which have become impregnated with oily substances and brown excretory pigment. Melin (1923) held that the ability of the mycelium of *Boletus* spp. to produce granules decreased with the length of time in culture and was only restored in symbiosis with conifer seedlings. No such decline with age has been noted in *B. elegans*; but a definite dependence on nutritive conditions has been observed. Papillation is slight in cultures on media with no carbohydrate, very few of the large brown granules being found; it increases in amount with increasing carbohydrate content, but at no time is it entirely absent.

The re-examination of the mycelium of *B. elegans* has disclosed the existence of a greater similarity between the mycelia of this and other species of *Boletus* than had been suggested by Melin. All the species so far grown in culture show three features in common, simple branching, 'paarige' branching, and papillated hyphae; many also exhibit clamp connexions and brown aerial hyphae—*B. elegans* is no exception.

## IV. PHYSIOLOGICAL CHARACTERS

### General experimental procedure.

Certain difficulties connected with the standardization of inocula had to be overcome before trustworthy data for growth could be obtained. Not only are spore inocula unavailable, but hyphae alone will not grow, so that a

certain amount of substrate has to be included in each inoculum. Also the previous history of the mother culture seriously affects the rate of growth of the inoculum; at least two generations before the experimental one must be similar in every respect to ensure comparable results (see Appendix).

In the majority of the experiments the inocula were derived from oat agar cultures once removed from stock culture; on this medium (Bonar, 1924)

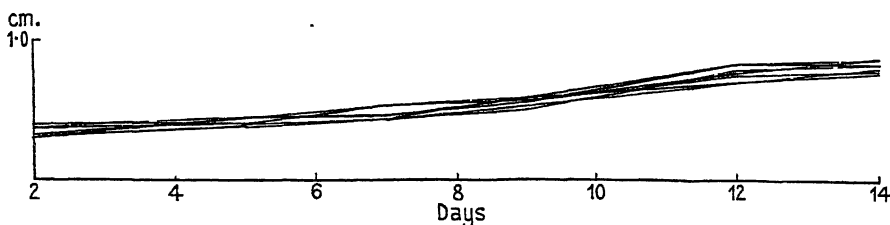


FIG. 9. Variation in growth rate of eight cultures on glucose silica gel.

growth was vigorous. Disc inocula, 0.4 cm. in diameter, were punched out from the growing margins of colonies about four weeks old. The average growth rate of 6–10 cultures of exactly similar origin was used as the basis of comparison in all experiments.

The estimation of dry weight was not a satisfactory method for measuring growth rate owing to the sensitiveness of the fungus to the poor aeration in liquid media. This necessitates a very shallow medium, and in consequence small cultures; matters were further complicated by the appreciable weight of the agar inoculum. On the other hand, the disadvantages of linear measurement as described by Brown (1922, 1923) did not hold in the case of *B. elegans* where the cultures were of very similar mycelial density on all the media used, hence this method was finally adopted. However, the irregularities in the shape of the cultures combined with their small size in the early stages rendered the direct measurement of increase in diameter somewhat inaccurate. In place of this a drawing was made of the image of the culture projected by an epidiascope and enlarged  $2\frac{1}{2}$  times. The area of the image was determined by a planimeter and the mean diameter was calculated from this value. The amount of variation obtained after the use of these methods can be gauged from Fig. 9, where are shown the individual mean diameters of a typical set of cultures when grown under exactly similar conditions.

The values of pH were determined colorimetrically; if 10 c.c. or more of a medium was available a Lovibond Comparator was used, if less a B.D.H. Capillator. Unless otherwise stated the experimental cultures were kept in an incubator with the temperature controlled at 20°–21° C.

#### *Concentration of the substrate.*

Styer (1930a) in his work on the mushroom found that the concentration of the nutrient solution influenced the rate of growth; this was retarded in solutions above 0.2 M. While no experiments have been carried out with the

express object of determining the effect of concentration on the growth of *B. elegans*, it became apparent that there is an upper limit of concentration above which growth is affected adversely. The exact position of this value was not determined.

The influence of concentration on growth rate was shown in two experiments among others. In the first experiment two 10 per cent. gelatin media of pH 5.4 were compared; their composition is shown in Table I.

TABLE I  
*Concentrations (Molar) of Media in Experiment 1*

Nutrients	Medium A	Medium B
Glucose . . . .	0.1	0.1
MgSO <sub>4</sub> ·7H <sub>2</sub> O . . . .	0.0004	—
K <sub>2</sub> SO <sub>4</sub> . . . .	—	0.0006
KH <sub>2</sub> PO <sub>4</sub> . . . .	0.007	—
Na <sub>2</sub> HPO <sub>4</sub> . . . .	—	0.087
NH <sub>4</sub> Cl . . . .	0.009	0.018
Citric acid . . . .	—	0.031
Total concentration . . . .	0.1164	0.2366

The average growth rates of the cultures on the two media are shown in Fig. 10; it is clear that growth was much better on medium A than on medium B. Since neither Mg-ions nor citric acid exert any effect on the growth of *B. elegans*, the presence or absence of these cannot be responsible for the result. The conclusion must be drawn that the lower concentration of medium A outweighs its poorer nutritive qualities, viz. less nitrogen and phosphate.

In the second experiment three glucose gelatin media were buffered at pH 5.0 with a citric acid-phosphate buffer (McIlvaine, 1921), added in three strengths, viz. full, three-quarter, and half strength. The resulting concentrations are shown in Table II.

TABLE II  
*Concentrations (Molar) in Experiment 2*

Medium	Nutrients	Phosphates	Citric acid	Total Concentration
I	0.12	0.086	0.037	0.243
II	0.12	0.064	0.028	0.212
III	0.12	0.043	0.018	0.181

From Fig. 11 it is seen that medium III with the lowest concentration gave the best growth. As far as nutritive qualities are concerned all solutions contained an excess of nutrients. Therefore one again concludes that good growth can only be obtained on media of low concentration.

These results, together with the knowledge that a concentration of glucose above 0.2 M always yields poor growth, leads to the conclusion that there is a limiting value for the concentration of the nutrient solution above which the growth of *B. elegans* is affected adversely. It is very probable that this



value lies between 0.18 M and 0.21 M, in which case it is of similar magnitude to that given by Styer for the mushroom. Whether this will be found to be a general characteristic of Hymenomycetes in pure culture remains to be determined; if so they are sharply distinguished from such fungi as *Aspergillus*

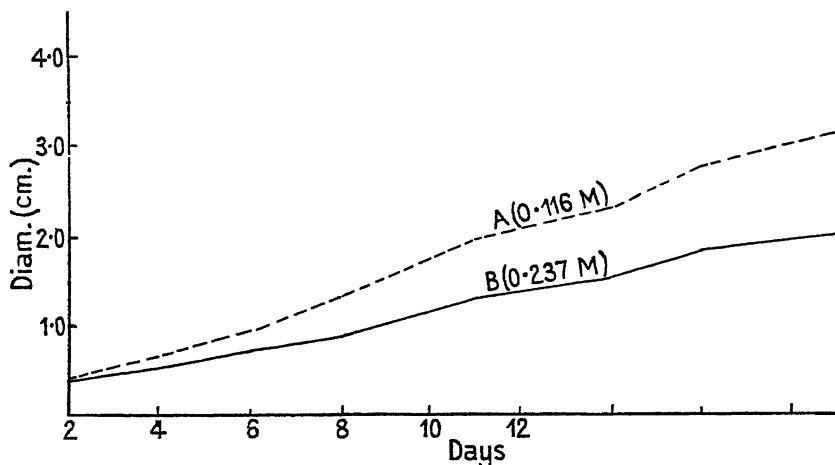


FIG. 10. Influence of concentration on growth rate—average diameters of nine cultures in A and of eight cultures in B (see Table I for detailed composition of media).

*niger* whose optimum concentration is above 1.0 M (Pringsheim, 1914). Presumably the explanation of this sensitivity to high concentration is to be sought in the osmotic requirements of the fungus; this problem, however, has not been pursued farther. For practical purposes it is sufficient to emphasize the need for considering the concentration of the medium when conducting experiments with soil Hymenomycetes in pure culture.

#### *Nutrient constituents of the substrate.*

Melin (1925) states with regard to possible carbon sources that the species of *Boletus*, including *B. elegans*, only grow well on glucose media. Although no exhaustive study of the carbon nutrition of the fungus was undertaken in the present investigation, certain classes of organic compounds were tested. It was found that while glucose is the best source of carbon for the English strain of *B. elegans*, nevertheless a number of relatively simple organic substances can also be utilized. Sugars such as xylose, fructose, sucrose, and maltose all yield good growth, as does also mannite. The starch of oatmeal is attacked with ease. Organic acids, however, such as tartaric, citric, and malic acids are valueless; nor can *B. elegans* use organic nitrogen compounds such as asparagine, peptone, gelatin, or nucleic acid as sources of carbon.

Since *B. elegans* is a forest soil organism it is important to know its reaction to substances such as pectin, cellulose, and lignin, which occur in varying quantities in the litter and the upper humus layers of the soil. Accordingly

the following materials were tested: cellulose in the form of filter-paper, xylum cellulose (product of *Bacterium xylinum*), ligno-cellulose materials such as larch litter of various ages and dead larch roots. In no case was growth possible, nor did penetration of the material take place.

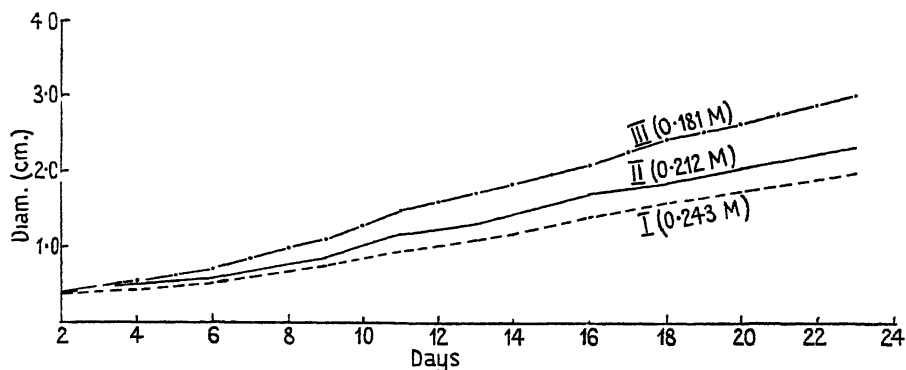


FIG. 11. Influence of concentration on growth rate. The diameter is that of ten cultures (see Table II for detailed composition of media).

When pectin, however, was supplied a very marked positive reaction was obtained. Melin's medium was used (see section II), the glucose being replaced by 1 per cent. soluble citrus pectin which had been precipitated with alcohol twice to remove all traces of sugar. The total dry weights of 10 cultures after 21 days were as follows: 1 per cent. glucose medium, 57.4 mg.; 1 per cent. pectin medium, 23.6 mg. Although growth was slower with pectin, it was very uniform and the cultures appeared particularly healthy, there being no evidence of the staling products which are always produced in unfavourable media. Tests for the presence of pentoses were positive, showing that hydrolysis of the pectin had occurred and that a pectinase was present. Since the insoluble protopectin also found in cell-walls cannot be extracted without hydrolysis to soluble pectin (Norman, 1937), it is impossible to determine whether the fungus can utilize pectin in this form.

The significance of the power of this fungus to utilize pectin is evident when one considers its natural nutritive conditions. There are two sources of pectic compounds in the soil, the litter and the living tree roots. The first is valueless to *B. elegans* since the pectic compounds in the litter are rapidly attacked by fast-growing bacteria and moulds (Waksman, 1927); the amount available in the humus for the higher fungi is therefore negligible. Preliminary investigations of young larch roots have shown, however, that pectin and pectic compounds are present in the middle lamella; these would certainly constitute one source of energy for a fungus such as *B. elegans* which forms a mycorrhizal association of the ectotrophic type. It may even be that the presence of pectic compounds in the young root is a help to its penetration by the fungus in mycorrhiza formation.

A short investigation of the nitrogen nutrition confirmed Melin's observations that ammonium compounds are by far the best nitrogen source for *B. elegans*. A comparison of the growth rates on  $\text{NH}_4\text{Cl}$ ,  $\text{KNO}_3$ , and asparagine in a silica-gel medium is shown in Fig. 12. The preference for

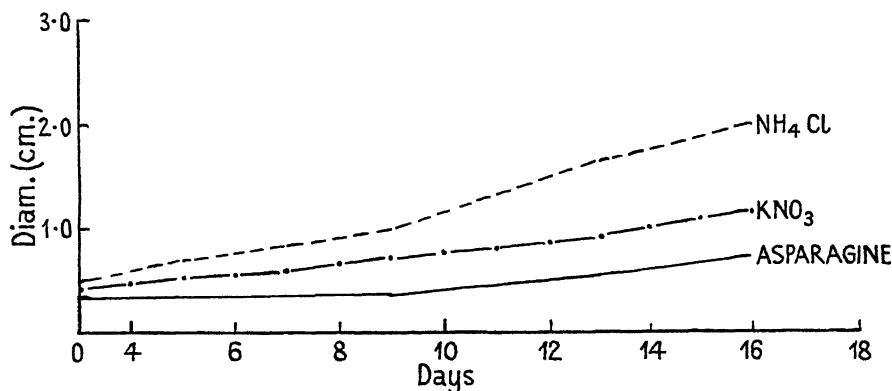


FIG. 12. The relative value of  $\text{NH}_4\text{Cl}$ ,  $\text{KNO}_3$ , and asparagine as nitrogen sources. (The diameter is the average of eleven cultures on each medium.)

ammonia is further emphasized by the rapid increase in acidity resulting from growth on  $\text{NH}_4\text{NO}_3$ . In addition *B. elegans* can liquefy gelatin and utilize peptone and nucleic acid derived from yeast. Melin (1925) stated that all true mycorrhizal fungi were limited to the ammonium salts of inorganic acids and certain protein compounds for their supply of nitrogen. *B. elegans* appears to be typical in this respect, growing most vigorously in pure culture in the presence of ammonium compounds.

As far as nutrition in pure culture is concerned, *B. elegans* prefers the simpler carbon and nitrogen sources, its preference for pectin being the only indication of its specialized habit; presumably, however, it shares this characteristic with other mycorrhiza formers among the species of *Boletus*.

#### *Range of pH on artificial media.*

The pH range of soils carrying larch in the Alps varies from acid to slightly alkaline values (pH 8.0); it is not known whether *B. elegans* occurs throughout this range in its native habitat. In this country, however, the writer has not observed the occurrence of *B. elegans* in soils above pH 6.0. It therefore seemed important to know something of the behaviour of this fungus with regard to pH value.

The desirability of using a solid medium led to the use of silica gels, since neither gelatin nor agar are possible with very acid values. In the preparation of the gels the method outlined by Styer (1930) was used as a basis. A suitable potassium silicate solution of molecular ratio  $\text{K}_2\text{O} : \text{SiO}_2 = 1.36$  was obtained from British Drug Houses Ltd. and, after dilution to three times the original

volume with distilled water, it was added to a 0.2 M phosphoric acid solution in which the nutrients had been dissolved. The correct proportions of silicate and phosphoric acid which give a gel setting in about twenty minutes were determined by trial. After placing 10 c.c. of gel in each dish and allowing it to set for six hours, a given pH was obtained by leaving 10 c.c. of a citric

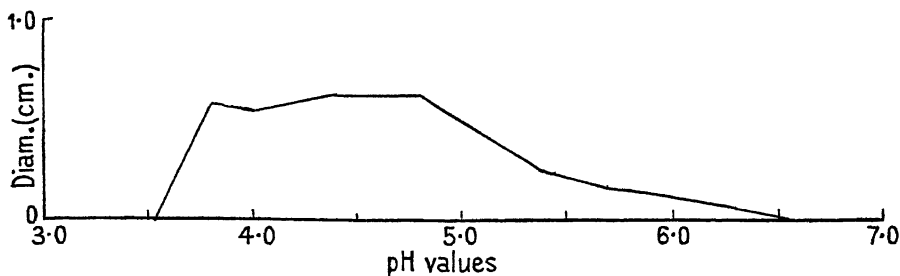


FIG. 13. pH range with  $\text{NH}_4\text{NO}_3$  as nitrogen source.

acid solution of the proper concentration in contact with a silica-gel plate for twelve to eighteen hours to attain equilibrium. After determining the pH value the supernatant liquid was poured away. The plates were sterilized by autoclaving for twenty minutes at 15 lb. pressure; the formation of bubbles in the plates during sterilization is prevented by the use of boiled distilled water for all solutions.

Two series of experiments were carried out, using  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  respectively. The compositions of the media were as follows:

	Concentrations (molar)
Glucose . . . . .	0.1
$\text{NH}_4\text{NO}_3$ or $\text{KNO}_3$ . . . . .	0.004
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . . . . .	0.0004
Potassium phosphates . . . . .	0.037
Citric acid . . . . .	0.04–0.005
	(varies with the pH value)
$\text{SiO}_2$ (hydrated). . . . .	2% approx.

The pH values of the medium at the margin of certain cultures were determined at intervals during growth. On the  $\text{NH}_4\text{NO}_3$  media the removal of the  $\text{NH}_3$  resulted in an increasing acidity which was appreciable after nine days; the average diameters of the cultures on the ninth day were therefore used as the basis of comparison. From Fig. 13 it is seen that growth with this nitrogen source is possible between pH 3.2–3.4 and 6.4; the region of optimum growth lies between pH 3.8 and 4.8. On the  $\text{KNO}_3$  medium the average diameter after fifteen days gave the truest comparison, since there was no appreciable change in pH value during that time and the growth-rate was very slow on such a poor nitrogen source as nitrate. In this series there were no cultures with a pH value below 3.2; at this value, however, growth is

extremely slow, so that the minimum value must be only just below it. The pH range on the  $\text{KNO}_3$  media is therefore pH 3.0–3.2 to 5.6–6.2 (Fig. 14).

The greater toleration of alkalinity on the ammonium medium is easily explained on the basis of a quick change towards more acid values enabling the fungus to tolerate a higher pH at the start; the reverse effect occurs at

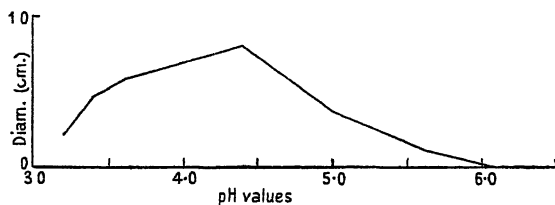


FIG. 14. pH range with  $\text{KNO}_3$  as nitrogen source.

the acid end of the range, where a tendency towards increasing alkalinity enables the inoculum to germinate on a slightly more acid substrate.

Considering the two series together one concludes that the maximum range of pH values possible for growth in media containing glucose, salts, and inorganic nitrogen compounds is pH 3.0–3.2 to 6.4. On the alkaline side this range is distinctly less than that found by Melin (1925) for *B. luteus*, *B. variegatus*, and *B. badius*, and the optimum at below pH 4.8 is more acid. Nevertheless, there is no evidence that *B. elegans* differs fundamentally from other Boletus species in its reaction to pH value.

## V. DISCUSSION

The present laboratory study of *B. elegans* in pure culture has furnished no clue to the factors concerned in its peculiarly specialized relation to the larch. Thus the examination of the mycelium has shown it to be morphologically very similar to that of other species of Boletus previously described by Melin. Furthermore, physiological investigation has not so far revealed any striking peculiarities; all the species of Boletus observed in pure culture have an acid range, with optimum growth on glucose and ammonium salts—*B. elegans* is no exception. With regard to the deleterious effect of high concentrations, the behaviour of other species of Boletus is not known, but it is very likely that they would exhibit a similar reaction. One is forced to the conclusion that further detailed studies of this nature will not prove of much value in elucidating the problems of specialization.

It remains to consider the physiological characters of *B. elegans* in relation to its habitat as a soil fungus; this raises the whole question of the validity of drawing conclusions from pure culture work and applying them to conditions in the field. Winogradsky (1935) has shown how misleading this can be in the case of *Azotobacter*. Thus observations of *B. elegans* in pure culture lead to the conclusion that it can only live in an acid soil, upon pectin and

simple sugars such as glucose, and with ammonium salts as nitrogen source. But in association with other soil micro-organisms the fungus may be able to tolerate a much wider range of conditions. For example, it is very unlikely that *B. elegans* has a wider pH range in the soil than that found in pure culture when optimum growth conditions are available; but that does not rule out the occurrence of *B. elegans* in soils whose pH value is outside this range, since the protective action of other organisms is possible.

Again, in a forest soil the amounts of pectin, starch, and sugars available for a slow-growing organism such as *B. elegans* in competition with bacteria must be practically negligible, so that it cannot be upon these that the fungus lives. For while it is safe to infer from pure culture work that *B. elegans* can use simple carbon compounds if available in the soil, it is not proper to conclude that the inability to utilize more complex substances in pure culture will also show itself in the soil. The fact that no evidence has been obtained of the breakdown of cellulose or lignin under laboratory conditions does not exclude these substances as possible carbon sources in the soil, though it renders it unlikely. It is more probable that *B. elegans* uses some degradation products of these materials released by other organisms. But until such substances are available for pure culture work no test of this view is possible.

The following considerations suggest that *B. elegans* normally lives in a state of semi-starvation upon traces of food materials. Firstly, growth in once-steamed soil is similar to that in natural conditions, with very fine hyphae ramifying between the particles; but the addition of even a trace of glucose results in an immense increase of growth which can be further increased up to a level of 0.007 gm. of glucose per gram of soil; still larger quantities of glucose, however, produce no further increase in growth. Secondly, in the soil the hyphae are only sparsely papillated with brown granules; increased quantities of available carbohydrate, however, both in soil and agar cultures always result in a marked increase in the papillation of the hyphae. It is also of significance in this connexion that the hyphae of *B. elegans* on the outside of the mycorrhizal mantle are often covered with similar brown granules, suggesting the presence of an addition to the carbon nutrition of the fungus when in association with the root. It is tentatively suggested that this may be the role of the pectic compounds in the cell-walls of the young root. The evidence for this will be considered in a later paper.

The conclusion is drawn that a laboratory study of this type, while an essential preliminary, cannot produce a solution to the problem of the behaviour of a soil fungus such as *B. elegans*; that will only be achieved after a study of the organism in relation to other members of the soil flora and the higher plant.

## VI. SUMMARY

1. The mycelium of *B. elegans* is described and compared with previous descriptions. There is no evidence that the mycelium differs in any important respect from that of other species of *Boletus*.

2. The growth of *B. elegans* is affected adversely by concentrations greater than about 0.2 M.
3. In pure culture *B. elegans* can use sugars, starch, and pectin, but not cellulose or lignin.
4. While nitrate, asparagine, peptone, and gelatin can serve as nitrogen sources, optimum growth is only obtained on inorganic ammonium salts.
5. The maximum pH range on media containing glucose, salts, and inorganic nitrogen compounds lies between pH 3.0–3.2 and 6.4.
6. It is concluded that the method of pure culture is only of limited value in the study of a soil fungus such as *B. elegans*.

I wish to express my indebtedness to Dr. M. C. Rayner for suggesting this problem, to Professor W. Neilson Jones under whose direction it was carried out, and to both of them for valuable advice throughout the progress of the work.

#### APPENDIX

Melin (1925) notes that the growth rate of *B. elegans* is affected by the age of the cultures when used for inoculation. Two experiments show that this effect may extend over more than one generation and that in consequence its importance in pure culture work is even greater than Melin suggested.

*Expt. I.* Dishes of Melin's glucose agar were inoculated with disc inocula whose past history is shown below. The period given is the age of the culture when it supplied the inoculum for the next.

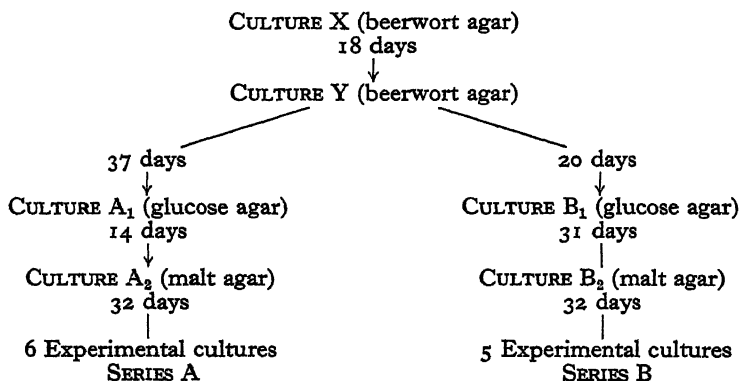


Fig. 15 shows that the growth-rate of series A was greater than that of series B. It is probable that the inocula from culture  $A_1$  used to start culture  $A_2$  contained less staling products than the corresponding inocula from culture  $B_1$  used to inoculate culture  $B_2$ . Apparently the initial greater quantity of staling products in  $B_2$  resulted in less vigorous growth with a corresponding decrease in the growth rate of inocula derived from  $B_2$ .

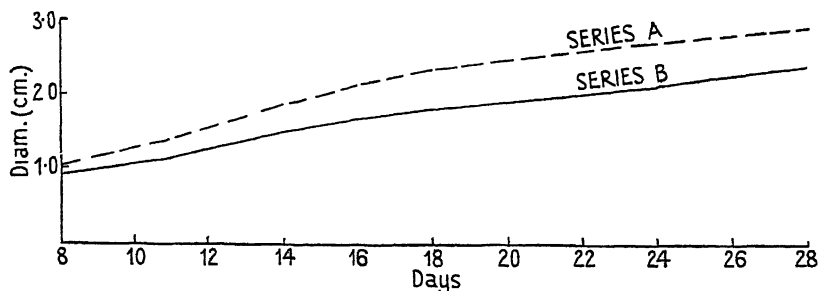


FIG. 15. The effect on growth of the past history of the mother cultures. For details see p. 148.

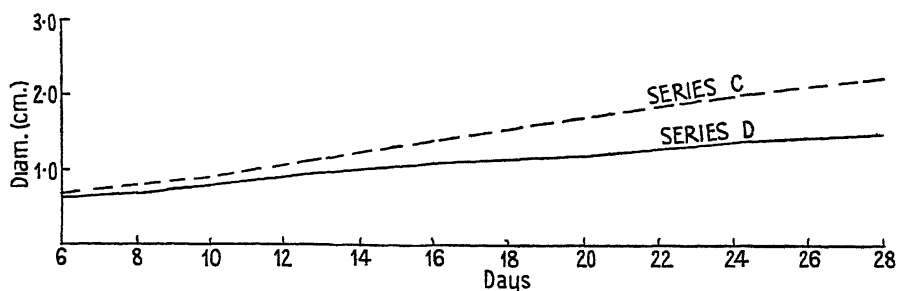


FIG. 16. The effect on growth of the past history of the mother cultures. For details see below.

*Expt. II.* The medium was similar to that used above, but the glucose was replaced by levulose. The history of the cultures was as follows:

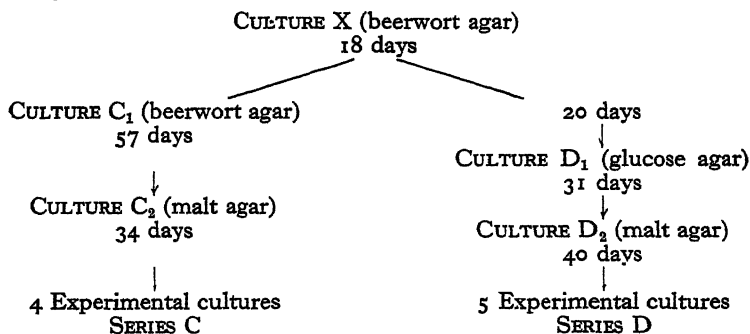


Fig. 16 reveals the marked difference between the two sets of cultures: series C grew more vigorously than series D, although culture  $C_1$  was 57 days old when used and culture  $D_1$  was only 31 days old. It appeared to be of more importance that culture  $C_2$  was only 34 days old when used while culture  $D_2$  was 40 days old. In this experiment the deciding factor appears to have been the smaller quantity of staling products in the younger culture which



masked the effects of its previous history. It should, however, be noted that culture D<sub>1</sub> was on glucose agar, while C<sub>1</sub> was on beerwort agar, which is generally a better medium for growth than glucose agar. It is therefore probable that the deleterious effect of the greater age of C<sub>1</sub> was partially overcome by the more vigorous growth possible on beerwort agar.

Considering the two series together, it is clear that at least two generations prior to the experimental one must be similar if comparable results are to be obtained. That this effect is largely attributable to staling products is evident from the fact that a greatly increased growth rate results from inocula which have been leached in sterile water. In conclusion it should be emphasized that it is not possible to explain these results completely, without assuming that the influence of staling products extends not only to the first but also to later generations.

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# Growth Rates and Water Loss in *Cladium Mariscus* R.Br.

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With seven Figures in the Text

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## I. INTRODUCTION

PREVIOUS publications (Conway 1936*a*, 1936*b*, 1938) have already given some account of the behaviour of this species in the field, and an attempt has been made to show how the anatomy of the plant is correlated with the habitat conditions under which it will flourish.

Special attention was paid to the growth rates of the leaves, but the subject was treated entirely from the ecological point of view, that is, the growth rate was taken as an indicator of the favourable or unfavourable conditions which are offered to the species by any particular habitat. The present aim is to consider the available data from the physiological point of view, and to describe the results of laboratory experiments which were carried out in order to verify or amplify any hypothetical interpretation which it might be possible to give to the field observations.

The mature plant of *Cladium Mariscus* has a short underground rootstock and around its growing-point new leaves are produced until the time of flowering of the shoot, when the scape bearing the inflorescence grows out, and puts an end to the further development of that axis. As in many other Monocotyledons, the meristem of the leaves is basal, so that the growing leaves have a fully differentiated lamina separated from the stem axis by a block of meristematic tissue in which the only differentiated elements are a few slender protoxylem and protophloem elements which connect the completed vascular bundles above and below. *Cladium* represents a rather unusual type in that the inner group of actively growing leaves appears to behave

as a physiological unit, since each leaf increases in length by precisely the same amount as the others over any given time period.

The extension in length is brought about by the zone of cells at the upper end of the block of cells which are actually dividing and lies between these

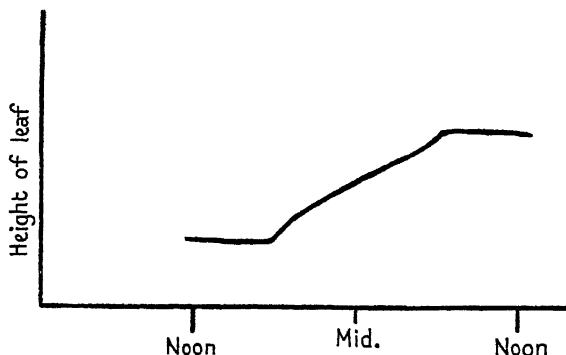


FIG. 1. Diagram to illustrate the typical form of auxanometer record obtained in the field, for the vertical extension of the growing leaves of *Cladium Mariscus* during a 24-hour period in fine summer weather.

and the differentiating cells above. Throughout this paper it is the phenomenon of cell extension that is discussed, and not that of cell division; for this there are two reasons. Firstly, the increase in length of the leaf due to cell division is negligible compared with that due to cell extension. Secondly, the available data on cell division frequency suggest that rate of division and rate of extension are not always closely correlated, if short time intervals of the order of a few hours are considered. For instance, at midday on a hot day when the extension rate may be zero, there are as many nuclear divisions occurring in the meristem as there are during the night when the extension rate is at a maximum.

Since the extension rate is the same at any instant for all except the oldest of the extending leaves, it is a simple matter to make observations on it. Continuous records in the field can be obtained by attaching an auxanometer to any one of these leaves. Such records were taken during 1935 and 1936 (Conway, 1938), and the most important results can be summarized in relation to the diagram given in Fig. 1. This shows an idealized curve for the variation in height of a leaf during twenty-four hours in fine summer weather; the extension rate is given by the slope of the curve at any instant. The main features of the results are as follows.

1. Extension is inhibited during the hours of days when the leaves of the plant are exposed to full sunlight, so that there is a clear division of the twenty-four hours into a 'growing period' and a 'non-growing period'. Abundant evidence exists to show that this inhibition is correlated with high

light intensity incident on the leaves, since overclouding of the sky, whether for part or for the whole of a day, leads to a positive extension rate of the leaves during the overclouded period.

2. In the hottest weather, during the middle of the day, not only is extension inhibited, but shows a small negative rate, that is, the leaves are shrinking slightly.

3. The change from a low to a high extension rate and the converse can take place with remarkable suddenness, being often completed within  $\frac{1}{4}$  hour, as nearly as may be judged from the close time-scale of the auxanometer records.

4. Very commonly the curve is concave towards the time axis over most of the night hours, since it exhibits a higher extension rate at the beginning of the night's growing period.

5. Rather less commonly, there is a renewed increase in rate at the end of the night, or rather in the early morning. The extent to which this feature is apparent varies from plant to plant, but it is always most marked on those mornings when there is a very rapid rise of temperature from the low night values. Even when there is not an actual rise in extension rate, the records show a straight line during the latter half of the growing period, that is, the fall of rate from the initial high value does not continue throughout the night. Very frequently the phase of initially higher rate is not well marked either, and in that case a constant rate is maintained throughout the night. The cause of the early morning rise in rate has not been investigated experimentally and it has therefore not been considered worth while to discuss it in an hypothetical way.

6. The average extension rate over the growing period is closely correlated with the maximum air temperature of the preceding day.

All these points, with the exception of No. 5, could be interpreted on the hypothesis that the extension rate is determined by the difference between the suction pressure exerted by the cells in the zone of extension and the hydrostatic tension existing in the protoxylem strands passing through that zone. The tension in the protoxylem will be determined, to a first approximation, by the rate at which water is being lost from the mesophyll cells in the upper, fully differentiated part of the lamina which is exposed to the atmosphere. This rate of loss will be determined, according to the hypothesis, by the degree of opening of the stomata, which is itself determined by the incident light intensity. The suction pressure of the extending cells is assumed to depend largely on the supply of osmotically active solutes, and the results quoted under heading No. 6 above suggest that this supply is governed mainly by the photosynthetic activity of the aerial part of the plant during the previous day. If this were not the case, and the supply came from accumulated carbohydrate reserves, it would be surprising that the height increase per twenty-four hours should show such large fluctuations from one day to another, following the changes of weather conditions.

## 2. EXPERIMENTAL METHODS

A few preliminary experiments were performed in the laboratory at Westfield College, London, during June 1937, without any special methods for controlling temperature and humidity. Most, however, were done during the following July and August in the Botanical Laboratory of Cambridge University, where it was possible to use special chambers for the control of these conditions. One of these chambers was adapted only for high humidities, the other could give any desired degree of saturation between 40 per cent. and 100 per cent. Both were rectangular in shape, about 3 ft. 6 in.  $\times$  2 ft. in area and 2 ft. 6 in. high. One of the longer sides was made up of the two glass doors. The chambers were illuminated by lamps shining vertically down through the glass roof, over which was a constantly running water screen. A fan at one end revolved behind the grid of wire coils which served for heating the chamber, while the humidity was increased or maintained by a steam blower which entered the opposite end of the chamber. Temperature was controlled in both the chambers by a toluol bulb actuating a mercury relay. Humidity was controlled in the 'damp' chamber by an additional toluol bulb covered with wet gauze and suitably connected electrically with the circuit from the other bulb. In the chamber used for lower humidities the control was effected by a type of hair hygrometer, and here, instead of a steam blower, use was made of a calcium chloride air-drier, over which the circulating air current was made to pass.

It was found impossible to prevent the slight temperature changes which occurred when the lamps were switched on or off; these changes were anything up to 1° C. in magnitude and produced corresponding alterations in the percentage saturation of the atmosphere. Throughout this account, therefore, temperatures and humidities are given as the ranges in which the experimental conditions lay, or sometimes, for the sake of brevity, as the mid-point of the range. Difficulty was also experienced in experiments where it was desired to maintain 100 per cent. saturation, since the chamber was not completely airtight, and the continuous use of the steam blower made it impossible to maintain a steady temperature. To overcome this, a fine spray of water was blown out from a small scent spray suspended in the chamber and connected by rubber tubing with a bulb worked by hand from outside. This spraying was carried out whenever the hygrograph in the chamber started to register values below the maximum. In such experiments the humidity range is classified as 99–100 per cent.

The plants used had been transplanted from their natural habitat (Wicken Fen) and established in pots of wet peat some months previously, so that they were growing healthily at the beginning of the experiments, and continued to do so throughout. The pot containing the plant was placed in a metal container sunk in the floor of the chamber so that the roots of the plant could be kept submerged in water. The plant was placed in the end of the chamber

farthest from the fan and was illuminated by a 1,000-watt lamp; the other end of the chamber was lit by a 500-watt lamp. When another plant, or two others, were used in the same experiment, they were all placed at the same end and placed so that they should receive as nearly as possible an equal share in the illumination.

Three types of measurement were made.

1. *Extension rate.* Continuous records of the height of the growing leaves were taken by means of the auxanometers whose construction has already been described elsewhere (Conway, 1938). They were modified slightly for the present purpose in two ways. In the first place, the drum which carried the record sheet gave one revolution per day instead of one per week which had been the arrangement when the instruments were used in the field. Secondly, the leaf was attached to the auxanometer lever by sliding its edge carefully between two small pieces of cork which were held firmly together by a metal spring. This gave an attachment quite rigid enough for laboratory conditions and much easier to manipulate than the type of attachment used in the field. The auxanometer was set to give the maximum magnification of the height changes, since over periods of a few hours quantities of the order of 1 mm. were being observed. Usually only one plant was used to give growth records, but occasionally a second auxanometer was introduced and attached to another plant.

2. *Stomatal opening.* These measurements were made by a modified porometer method.

3. *Rate of water loss.* A third plant was placed in the chamber, with its pot wrapped in rubber sheeting tied securely round the base of the plant. The water loss was estimated by removing the plant from the chamber when desired, and weighing it on a balance which weighs to 0.5 gm. up to 5,000 gm. and is quick reading.

These three types of observation were not all carried out in every experiment. The third, in particular, was only performed rarely, because it involved opening the doors of the chamber, and even though this was done gently and quickly, and conditions returned to equilibrium within a minute or two, it was felt to be detrimental to the experiment as a whole.

Except for a preliminary group of 'exploratory' experiments, all the rest were performed on one main plan. This was, namely, to follow the effect of a period of darkness between two light periods, and to find how the effect varied with the degree of humidity of the atmosphere. Time was very scanty since the apparatus was only available for a period of five to six weeks, and this is why the experiments were so limited in design. For the same reason, it was not possible to try out variations in the light intensity which was used, nor the temperature which was maintained. The latter, namely 27° to 28° C., seemed undesirably high compared with natural conditions, but owing to the hot weather prevailing, it was not possible to achieve a temperature control at a lower temperature. No measurements were taken of the radiant energy

supplied by the 1,000-watt lamp, but it is likely to be small compared with sunshine of field conditions. For this reason, if a plant was not to be used on the following day, it was returned to a well-lit greenhouse at the end of a day's experiment. Further, if a plant was left in the chamber overnight no heat or electric light was supplied, and the doors of the chamber were left open to the air of the laboratory, so that the night conditions before an experiment might not be too far removed from what would be the normal conditions in the field. Light and heat were applied to the chamber while the necessary preparations and adjustments were being made before an experiment, and when the chamber had been closed, an interval of half an hour or more was allowed to pass before taking readings, to ensure that the temperature and humidity controls had been established and that the plants were not suffering large changes in conditions immediately before the start of measurements.

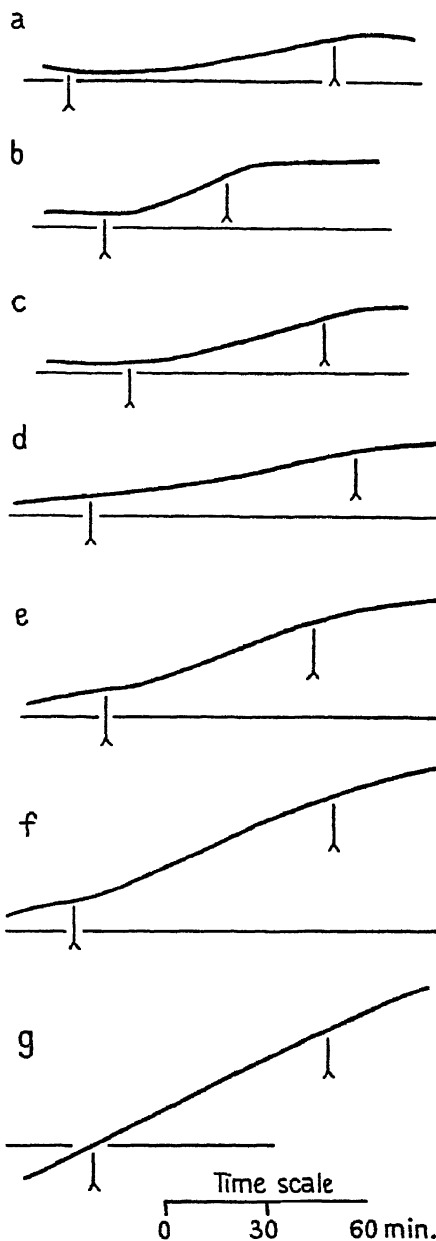
The same three or four plants were used throughout the work, and most of the results concern two of them only (referred to as plants A and B). However, in a previous publication (Conway, 1938), evidence has been put forward to show that conclusions may have some general validity for the species although based on experiments with individual plants.

### 3. EXTENSION RATE, LIGHT INTENSITY, AND HUMIDITY

Figs. 2 and 3 show the records of the extension curves obtained for plants A and B, and include all the experiments that are comparable, that is, all except a few preliminary experiments at high humidities which were not carried out on the more or less standardized procedure of the remainder, and in which the control of conditions in the chambers was more imperfect. Each graph starts in the first period of illumination of the plant; the first arrow indicates when the lamps were switched off, the second when they were switched on. The variation in length of the dark period, more marked in the case of plant A, came about because of the porometer readings that were going on at the same time. It was sometimes of interest to follow the behaviour of the stomata over a longer period than usual if they did not appear to be showing a steady drift in one particular direction. It is not considered to vitiate the comparison between the curves, since the extension rate in the dark period appears as a general rule to be maintained at a steady value, after the initial change.

The curves in the figures have been copied as accurately as possible and are all drawn to the same scale. The extension rates represented here vary from  $-0.1$  to  $0.6$  mm. per hour. The experiments are arranged from above downwards in order of ascending humidity, and other information is given beside each curve.

Considering first of all the slopes of the curves during the periods of illumination, it is apparent that they increase progressively, from slightly negative values at the lowest humidities, to values which are equal, or almost



Expt.	Humidity range %	Zero hour of expt.
46	50-52	10.20
45	60-62	14.45
42	69-71	10.0
52	76-80	11.45
39	92-97	15.30
40	94-97	10.25
41	99-100	10.15

FIG. 2. Auxanometer records for experiments with plant A. Vertical lines indicate the limits of the dark period. The extension rates involved range from  $-0.1$  to  $0.6$  mm. per hour.



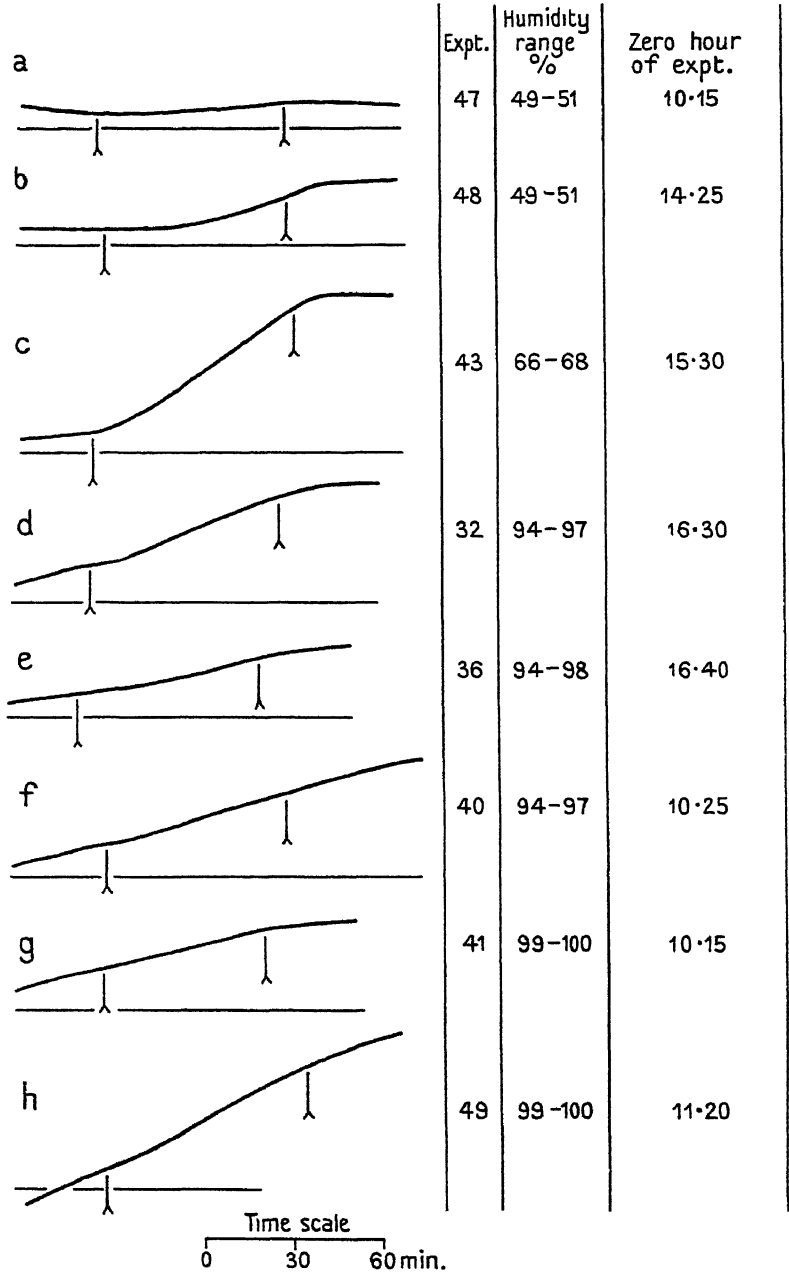
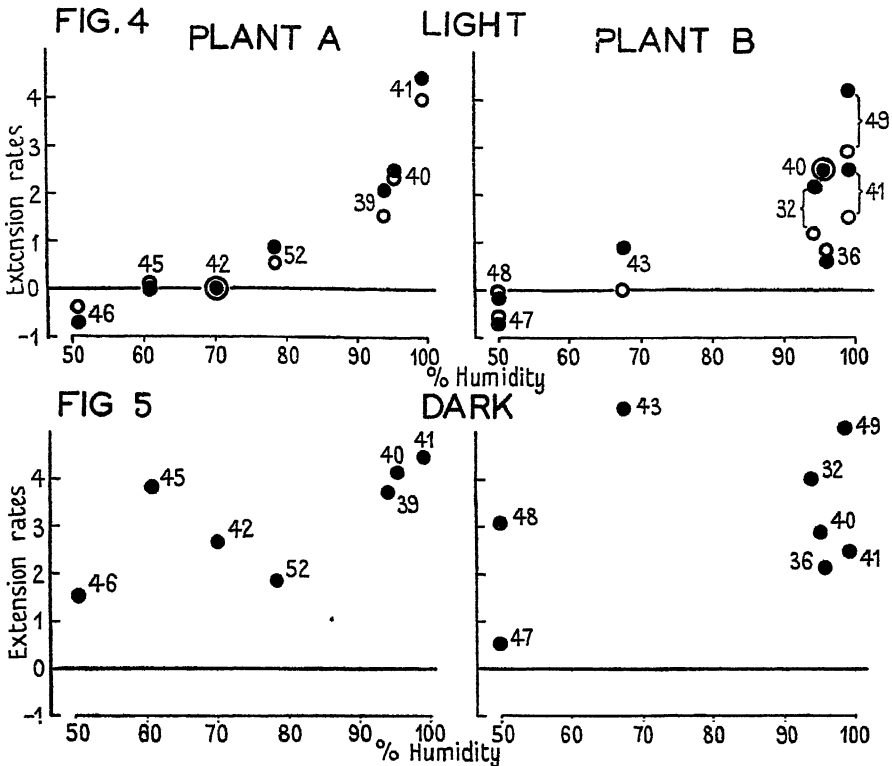


FIG. 3. Corresponding data to those of Fig. 2 for plant B.

equal, to the values for the dark periods at the highest humidities. Fig. 4 shows this result graphically. Ordinates represent the extension rates calculated from the angle of slope, abscissae the atmospheric humidity for each experiment. Black circles give the data for the first light period, the rate being



FIGS. 4 and 5. Fig. 4. Extension rates during the light periods at different humidities, in arbitrary units. Black circles, rate in first light period. Open circles, rate in second light period. The numbers are those of the experiments. Fig. 5. Extension rates during the dark periods, corresponding to the data in Fig. 4.

calculated from the region of the curve just preceding the dark period. Rates for the second light period are calculated from the latest part of the curve where the slope is constant or almost constant, and are represented by open circles. The number of the experiment is attached to each pair of points to make it easier to compare Figs. 2 and 3 with Figs. 4 and 5.

The data for plant A in Fig. 4 show a regular variation with humidity, and those for plant B are very similar, though more incomplete and irregular. Fig. 5 shows the extension rate during the dark period of each experiment, or, more precisely, the rate during the later part of the dark period when it has become constant. In this case the rates fluctuate markedly and without special relation to the humidity, suggesting that the extension rate in the dark is not

controlled primarily by varying rates of water loss but by other more or less unknown factors. These factors are presumably concerned with variations in the treatment of the plant prior to the experiment; such variations, although minimized, could not be entirely avoided. The very high dark rate shown in expt. 43, for instance, may have been caused by the fact that the plant had been all morning in the warm well-lit conditions of the controlled chamber, since it was being used in another experiment.

On the other hand, some factor has produced in expt. 36 a very low dark rate, and it is therefore not surprising to find that the light rates in this experiment are also abnormally low, thus giving the apparently aberrant points in the graph for plant B in Fig. 4.

The data in Figs. 2 to 5 show that, broadly speaking, the extension rates in the light approach more and more closely to those in the dark the nearer the humidity approaches saturation point, and in expt. 41 for plant A (Fig. 2*b*) it has been possible to neutralize entirely the effect on the extension rate which at lower humidities would normally be produced by a change from light to darkness. For the change from darkness to light in this experiment, and for the other experiments at the highest humidities, the effect is not entirely obliterated. This might be due to the difficulty of obtaining a genuinely saturated atmosphere, but in any case it does not conflict with the view that extension in the light is limited only by transpiration, since it was shown by Darwin (1914) that leaves might still transpire in an atmosphere at 100 per cent saturation owing to the internal leaf-tissues having a higher temperature than the atmosphere.

The results therefore suggest strongly that in natural conditions the extension of the leaves is inhibited during the hours of bright sunshine by loss of water from the transpiring regions of the leaves, but will take place if water loss is minimized either by an increased humidity or by the lessening of the stomatal aperture which is brought about, as will be shown, by lowered light intensities.

Fig. 4 indicates that the point for zero rates of extension lies between 60 and 65 per cent. saturation. Below this, the rates are negative, that is, the leaves as a whole show shrinkage. No data are available concerning the humidity of the air in the natural habitat of the plants investigated, Wicken Fen, but it is likely that on hot days the relative humidity might fall below 60 per cent., giving the negative extension rates that are found in the field auxanometer records. The upper parts of the *Cladium* leaves in the sedge vegetation form the exposed surface of the plant community, and as Yapp (1909) has shown, the conditions in the air above this surface are much more favourable to rapid transpiration than those at a level lower down, within the actual strata of the vegetation.

#### 4. RATES OF WATER LOSS

On account of the large size of the plant, and the necessity for keeping its roots well supplied with water, a potted plant weighs 4 to 7 kg., so that it is

practically impossible to measure a loss of weight of less than  $\frac{1}{2}$  gm., even with the most sensitive balance available. This means that unless the rate of transpiration is very high, the rate of water loss has to be taken as an average over a period of at least half an hour.

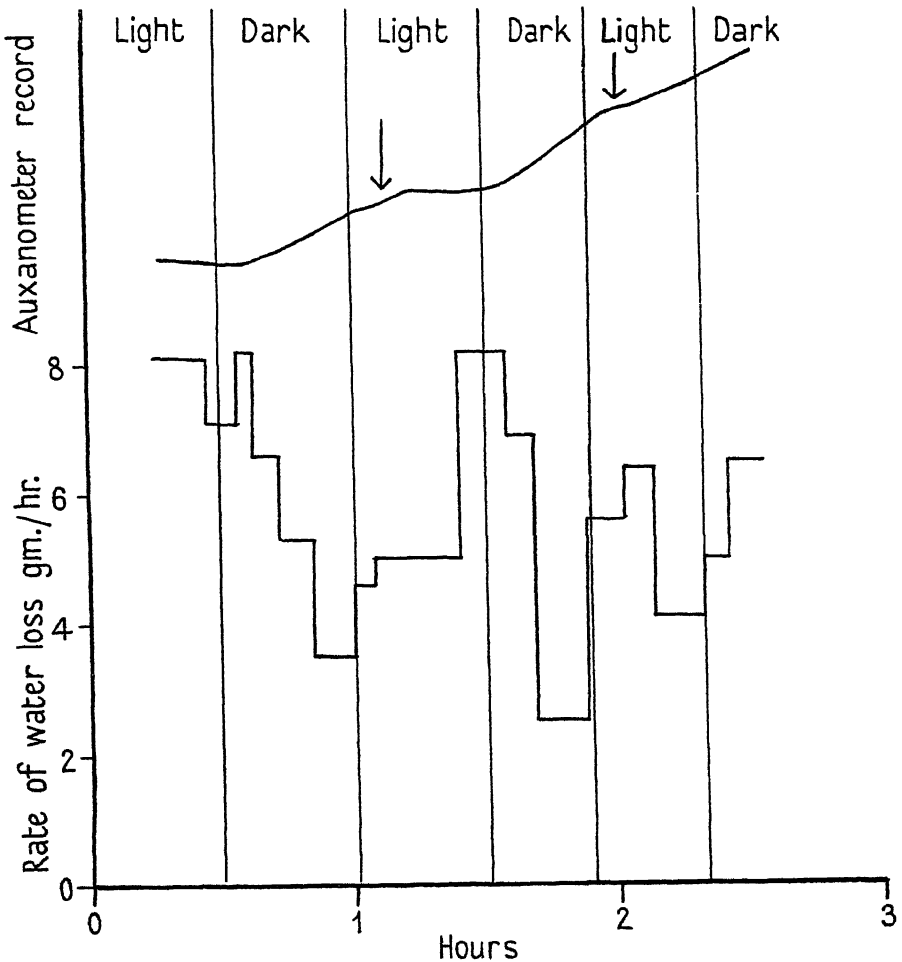


FIG. 6. Results of an experiment showing changes in extension rate and water loss on changing illumination. Direct sunlight was used as light source. Arrows indicate onset of periods of overclouding, which lasted seven minutes in the first case, more than thirty minutes in the second.

In one or two preliminary experiments, the plant was exposed to sunshine instead of to artificial light, and this raised the transpiration high enough to measure changes in water loss over periods of five to ten minutes. Fig. 6 gives the results of one such experiment. Above is shown the auxanometer record for plant B, while below are the rates of water loss given by plant A. It is not

possible to use one plant for both readings since the plant attached to the auxanometer cannot be moved. However, a repetition of the experiment with the plants interchanged gave similar results. It is shown by Fig. 6 that each of the changes of light intensity produces a change in rate of water loss by the end of seven minutes, and sometimes sooner, that is, in a length of time closely

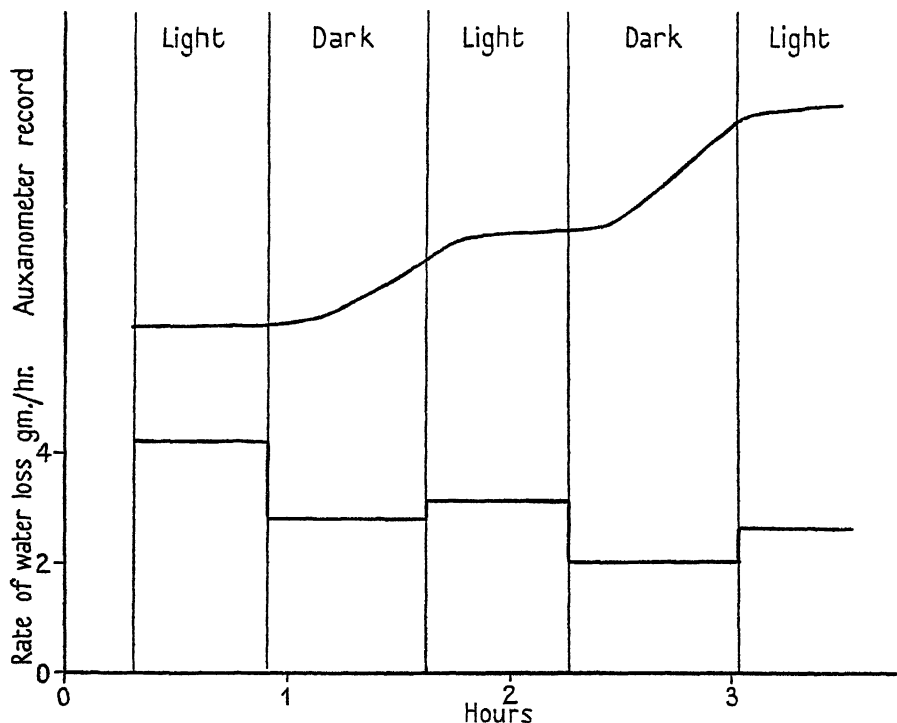


FIG. 7. Results of an experiment similar to that of Fig. 6, but using two 500-watt lamps light source.

comparable to that taken for the change in rate of extension. At the beginning of the experiment the sunshine was bright and the extension rate shows a slight negative value. During the next interval of illumination, however, the sun was temporarily obscured, as indicated in the diagram, and extension set in while this overclouding lasted. The method of measuring water loss is too crude to detect any change over such a short period. During the next period of illumination there was a renewed overclouding which persisted till the end of the experiment, and is correlated with an increase in the extension rate, as may be seen from Fig. 6. In this case it is possible to see a marked fall in the rate of water loss.

Fig. 7 shows corresponding results when the plants were illuminated by two 500-watt lamps, instead of by sunshine. Here the rate of transpiration was at a generally lower level and it was necessary to take average rates of loss

over longer periods. The lowered rate of loss in the dark periods is again apparent, though in addition there seems to be a general downward drift in rate. The extension rate changes are typical for the relatively dry atmosphere—that of the laboratory—in which these two experiments were performed. The observations on water loss in the experiments with the constant humidity chamber were somewhat meagre and unsatisfactory on account of practical difficulties such as, for example, the necessity of exposing the plant temporarily to the atmosphere outside the chamber in order to weigh it. As far as the results went they confirmed the negative correlation between extension rate and water loss which is exhibited by Figs. 6 and 7.

### 5. MOVEMENT OF THE STOMATA

A number of curves were obtained for the porometer readings taken during the experiments; the results varied from one experiment to another in a rather complex way. They are not discussed in the present paper since their interpretation does not bear directly on the problem of the growth-rate changes. The important result from the latter point of view is that in all cases the first reading of the porometer after switching off the lights showed a decreased rate as compared with the previous reading. Correspondingly, the re-illumination of the plants always produced an increase in the porometer rate. In some cases, by darkening the plant the rate was reduced to half its original value in two to three minutes; in others the rate was not immediately reduced greatly but showed a more gradual and long continued fall, the type of behaviour varying according to the position on the leaf at which the porometer was attached. However, the results in general showed that the stomata react to light-intensity changes in a space of time which is comparable with that which is involved in the growth-rate changes, and it is therefore reasonable to conclude that when growth-rate changes are induced by alterations in light intensity, this is due to the effect on transpiration rates of the altered stomatal apertures.

### 6. CONCLUSIONS

Perhaps the main interest of these results on extension rates and water loss lies in their relation to the anatomy of the plant and the restriction of habitat which is enforced by such 'physiological anatomy'. *Cladium Mariscus* is a plant which shows the effects of water deficit, even when its roots are growing in water, because the vascular supply to the leaves is so limited that not enough water can pass through to allow both vigorous transpiration and cell extension to go on at the same time. The same water deficit is made evident in another way, namely, in the xeromorphic character of the leaves. The presence of a thick cuticle, numerous bands of lignified fibres, marginal spines, and a waxy bloom on the surface suggests a restricted water-supply to the developing leaves.

When meristematic activity at the leaf-base ceases the vascular bundles

become fully differentiated to the base and become linked up with the vascular system of the stock and so with the roots. The 'bottle-neck' in the communication between the leaf and the rest of the plant is thus removed.

All the phenomena discussed here deal with the restriction to the flow of water resulting from the meagre cross-sectional area of the protoxylem. There is probably a parallel resistance offered to the passage of organic substances which presumably move in the phloem, for the protophloem in the meristematic region is as meagre as the protoxylem.

This 'bottle-neck' phenomenon must exist in many other monocotyledons where the leaf meristem is basal, yet these are not obligate halophytes. In *Carex arenaria*, for example, as in *Cladium Mariscus*, there is a group of young leaves all growing upwards at the same rate and supplied only by provascular strands, yet from the nature of its habitat it is clear that the mature plant, unlike *Cladium*, is not sensitive to lack of ample moisture in the soil. Where the key to this difference lies—whether, for instance, in a different ratio of growing to non-growing leaves, or in a different type of root development or of vascular connexion between root and leaf, to mention a few possibilities at random—remains for further investigation. An answer to this problem might have some bearing on the phylogeny and ecology of the Cyperaceae as a group.

I am much indebted to Professor F. T. Brooks and Mr. G. E. Briggs for permission to work in the Botany School, Cambridge, and to use apparatus that was already in working order. To Dr. E. J. Maskell my warm thanks are due for his kindness in reading through the manuscript and for his illuminating criticism and comments.

I have pleasure in acknowledging a grant from the Dixon Fund of London University, which met the cost of the sensitive balance and parts of the auxanometers used in these experiments.

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# Physiological Studies in Plant Nutrition

## X. Water Content of Barley Leaves as Determined by the Interaction of Potassium with certain other Nutrient Elements

### PART I. *The Relationship between Water Content and Nutrient Composition*

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### INTRODUCTION

IN previous publications from this Institute (Gregory and Richards, 1929; Richards, 1932; Gregory and Sen, 1937) it has been claimed that deficiency of potassium may lead to greatly increased succulence of foliage, a result which was recognized to be diametrically opposed to that obtained by certain other workers. For instance, Janssen and Bartholomew (1929, 1930) using a variety of plants showed 'quite conclusively that high-potassium plants are more succulent than low-potassium plants'. It was shown by Richards (1932) that this discrepancy could not reasonably be accounted for by the different responses to potassium of the species investigated, and attention was drawn to the very different constitution of the culture solutions used. The considerable differences between the type of plant investigated by Janssen and Bartholomew and others and that investigated here are not confined to water content, but are far reaching, embracing such characters as leaf colour (Richards, 1932), leaf size, meristematic activity, and carbohydrate content (Richards and Templeman, 1936; Russell, 1937, 1938), assimilation rate, ratio of roots to tops, and so on. It is thus abundantly clear that all



physiological work on the effect of potassium needs interpreting in relation to the concentration in the solutions of ions other than potassium.

In this paper attention will be confined to variation in water content as related to potassium nutrition. From this point of view papers of importance have appeared from two sources. James (1930), working with potatoes in the field, found greater succulence with increased potassium provided this was applied as chloride, but a slighter effect if applied as sulphate; the conclusion was reached that the observed effect is due to the anion, and not to potassium. In a later paper (James, 1931) the relationship of potassium to succulence is discussed more fully, using experimental data obtained by sampling methods from plants grown at constant potassium level; the internal changes of concentration and movements of potassium during growth were observed and correlated with water content and certain growth measurements. It was concluded that the higher osmotic pressures associated with higher internal concentrations of potassium must increase the water content, but that this is not 'the only or even the principal mechanism at work. Since potassium is readily soluble, it is clear that the presence of water may be a cause as well as an effect of the presence of potassium ions, and a truer picture is to be obtained by considering both effects. The close numerical relationship between the concentrations of the two substances is the expression of an equilibrium between them, not of the fluctuations of a dependent and an independent variable.' He claims that there is an equilibrium value lying somewhere between 0.5 and 1.0 per cent. potassium, so that an increase in the amount of potassium above this value results in the retention of more water, and vice versa. Again, 'the relation between potassium and water in the plant or whole organs is so important as completely to overshadow any connexion between potassium and total solids'.

The latter conclusion is deduced from the fact that in the experiment of James there was a very high correlation between potassium *content* and potassium *concentration* within the plant, and also a positive correlation between potassium content and water content; therefore there cannot also be a positive correlation between potassium and the amount of dry matter per unit weight of water, since this is the reciprocal of water content. This amounts to the assertion that potassium content and water content are highly positively correlated when both are measured in terms of dry weight, while potassium and dry weight are not positively correlated when both are measured in terms of water. That this is not necessarily confirmed by experiment is readily demonstrated from data collected by the author. Thus the data presented by Richards (1932) give no support to the claim, the correlation coefficients in the high potassium plants (series A) and in the very deficient plants (series D) being as follows:

	High K (A)	Low K (D)
$K_2O/D.W.$ and $H_2O/D.W.$	-0.227 (-0.480)	-0.125 (-0.658)
$K_2O/H_2O$ and $D.W./H_2O$	+0.841 (+0.791)	+0.268 (+0.852)

The figures in brackets represent the coefficients after the elimination of the linear term for age effect; those in heavy type may be regarded as significant. It is seen that a result diametrically opposed to that of James is obtained. The same data do not support in other ways the conclusion of James that there is an equilibrium value between the contents of potassium and water. As between treatments varying in potassium supply this is clearly not the case; for the average concentration over all leaves throughout life-history is 6.7 times as great in series A as in D, while in the latter half of the leaf series, when the effects of deficiency are most pronounced, this ratio rises to 14.6. Moreover, within both nutrient series the variability of the ratio  $K_2O/H_2O$  is much greater than the range 0.5–1.0 per cent. found by James, in series A the concentration of some leaves being 4–5 times as great as that of others, while series D shows a still greater range. In neither series is the variation of this ratio less than that of  $K_2O/\text{Dry Weight}$ , on the contrary in series A, the one comparable with James's data, the reverse is true.

These considerations do not, of course, imply criticism of the experimental results obtained by James, but they demonstrate effectively the uncertainties which arise when general statements of the function of potassium are attempted. The type of plant produced and the physiological response observed depend quite as much on the other ions available to the plant as on the absolute level of the potassium itself.

An attempt has been made by Warne (1936) to bring the present author's discordant observations on the effect of potassium on water content into line with the effects as more usually described, but in doing so he has patently glossed over the difficulties. He states that 'when the results are expressed on either the conventional fresh-weight or dry-weight basis, *apparent* variations in water content may be due as much to variations in dry matter as to *real* variation in water content'.

The concept of water content is quite definite and involves the ratio of two weights. In any given material it varies in time with variation in the absolute amount of either water or of dry matter present, or of both simultaneously. But when, as in all the experiments referred to by Warne, leaves from plants differently treated are compared, it becomes impossible without gross assumptions to analyse the observed differences of water content in terms of absolute differences of water and dry matter respectively; all that can be discussed with certainty is the ratio of the two. For varying treatment leads to variation of many kinds within the comprehensive fraction 'dry matter', altering the entire character of the structure, while the absolute weights and areas of corresponding leaves may also differ widely with treatment.

The particular measures which Warne apparently believes are appropriate for discriminating between 'apparent' and 'real' variations in water content are the weights of water and of dry matter both estimated on the basis of unit leaf area. No grounds for this choice are given, while serious criticisms of it may be raised. Indeed, results presented by Gregory and Richards

(1929), and particularly by Richards (1932), indicate strongly that between treatments differing in potassium and nitrogen supply the leaf area per unit dry weight is closely associated with water content, suggesting that as between these treatments leaf-volume and area are largely determined by the extent to which the cells are inflated with water; moreover, the contraction and expansion of leaves with variation in water content is a well-known phenomenon. Apart from complications of this kind, a further difficulty arises in that considerations of leaf thickness are ignored. If for instance a given treatment produces an increase in thickness of leaf without in any way affecting composition, then this treatment results simply in proportional increases in both the weight of water and of dry matter per unit leaf area. The water content, both as measured and as conceived on any rational grounds, remains the same, and it would clearly be fallacious to conclude that the increase in dry matter per unit area is masking an increase of 'real' water content. Yet this is precisely the interpretation Warne (1936, p. 408) puts on some of his data.

Apart from the doubtful validity of the argument employed, the fact remains that the experimental data of Gregory and Richards (1929) and of Richards (1932) could not be accounted for on the lines suggested; for as the tables and graphs of both dry weight and water per unit area there presented show conclusively, in these experiments the former *decreased* with reduction of potassium, while the latter *increased*, i.e. on Warne's interpretation 'real' water content increases with reduction of potassium supply just as does the 'apparent, water content, though to a lesser extent.

Warne concluded that increase of cell-size is the primary effect of the addition of potassium; this results in relatively larger vacuoles, leading to increased water content. Clearly the published results from this Institute cannot be accommodated to this view, for in the type of plant here investigated cell-size is almost certainly not increased by addition of potassium, and may be decreased. Cell-size must presumably be included among those characters which may vary with potassium supply, but whose direction of change is consistent only when the levels of other ions present are rigidly defined.

Previous researches therefore have thrown very little light indeed on the causes of variation of water content with potassium supply, and progress in this problem must involve more detailed investigation of the variations within the dry matter fraction. A contribution to such an analysis can be made from data collected in 1934 at Rothamsted in a comprehensive growth experiment dealing with the level of potassium supply in relation to the composition of the nutrient solution, and in fact a preliminary survey of the main results of this experiment has already appeared (Gregory, 1937). The descriptive aspect of the responses of water content to variation in nutrient conditions is dealt with in the first part of the present contribution; in the second part, the relation of these responses to the internal contents of metallic ions, &c., will be analysed and discussed.

## EXPERIMENTAL METHODS

A pure line barley, var. Plumage Archer, was again used in this experiment, which in all essentials followed the methods described in previous publications. The plants were grown in sand culture with the nutrient combinations shown in Table I; the nomenclature scheme adopted is given in Table II. The first six basal treatments were combined with each of the three potassium levels, but the last four treatments with only the K<sub>5</sub> dosage; thus in all there were twenty-two nutrient combinations. The basal nutrients were designed so as to allow of a wide range of values of the ratio Na/Ca being investigated, each value being combined with two levels of phosphorus, of which, however, the lower level was kept well above that of the reduced potassium levels. Nitrogen, magnesium, and chlorine were constant throughout, all unwanted variation being thrown on the sulphate radicle, which, according to James (1930), has but little effect on water content. Constant iron and manganese were also added throughout, while the initial pH of all solutions was adjusted by the addition of sulphuric acid to that of series C (high calcium).

TABLE I

*Nutritional Scheme*

## (1) Basal nutrients (gm. per pot)

	HA	LA	HB	LB	HC	LC	HD	LD	HE	LE
NaNO <sub>3</sub>	9.11	9.11	4.56	4.56	—	—	—	—	4.56	4.56
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	3.00	0.60	1.50	0.30	—	—	—	—	1.50	0.30
Na <sub>2</sub> SO <sub>4</sub> ·10H <sub>2</sub> O	—	2.16	—	1.08	—	—	3.38	3.38	9.97	11.05
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	—	—	6.33	6.33	12.65	12.65	12.65	12.65	6.33	6.33
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> H <sub>2</sub> O	—	—	0.528	0.106	1.06	0.211	1.06	0.211	0.528	0.106
CaCl <sub>2</sub> ·6H <sub>2</sub> O	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25

(2) Potassium levels, K<sub>2</sub>SO<sub>4</sub> (gm. per pot)

K <sub>1</sub>	1.85
K <sub>3</sub>	0.206 (i.e. $\frac{1}{9}$ K <sub>1</sub> )
K <sub>5</sub>	0.0229 (i.e. $\frac{1}{81}$ K <sub>1</sub> )

TABLE II

*Nomenclature*

	High sodium Low calcium.	Medium sodium Medium calcium.	No sodium High calcium.	
High potassium	HAK <sub>1</sub>	HBK <sub>1</sub>	HCK <sub>1</sub>	High phosphorus
	LAK <sub>1</sub>	LBK <sub>1</sub>	LCK <sub>1</sub>	Low phosphorus
Medium potassium	HAK <sub>3</sub>	HBK <sub>3</sub>	HCK <sub>3</sub>	High phosphorus
	LAK <sub>3</sub>	LBK <sub>3</sub>	LCK <sub>3</sub>	Low phosphorus
Low potassium	HAK <sub>5</sub>	HBK <sub>5</sub>	HCK <sub>5</sub>	High phosphorus
	LAK <sub>5</sub>	LBK <sub>5</sub>	LCK <sub>5</sub>	Low phosphorus
Low potassium	Low sodium High calcium.	High sodium Medium calcium.		
	HDK <sub>5</sub>	HEK <sub>5</sub>		High phosphorus
	LDK <sub>5</sub>	LEK <sub>5</sub>		Low phosphorus

Regarding nomenclature: phosphorus level is designated by high (H) or low (L), the latter being one-fifth the former. The letters A–E represent the Na/Ca ratios. Series A receives excess sodium and minimal calcium and is similar to the solution which has hitherto been used in our experiments; series C receives excess calcium and no sodium; while series B is intermediate. Series D differs from series C in that the requisite equivalent amount of sodium has been added to replace the difference of potassium between levels K<sub>1</sub> and K<sub>5</sub>, i.e. HDK<sub>5</sub> differs from HCK<sub>1</sub> in that 80/81 of the potassium of the latter has been replaced by equivalent sodium. Finally series E receives the same salts as series B except that an excess of sodium has been added, bringing its sodium level up to that of series A.

Samples from all treatment were taken on three occasions during the growth period, and again at harvest. The sample from each treatment consisted of three replicate pots, containing nine plants in all; owing to the large amount of material to be dealt with at one time each sample was spread over three days, one pot from each treatment being taken each day. The sampling dates were as follows:

Sample 1. June 12–14, at 32–34 days from germination.

„ 2. June 19–21, at 39–41 „ „

„ 3. July 3–5, at 53–55 „ „

Observations made at each sample included fresh and dry weights of leaf laminae and ‘stems’ (i.e. true stems, expanded leaf bases, and young leaf tissue still within the sheath of the last expanded leaf), dry weights of dead leaf and root, tiller numbers, and leaf areas with a view to determining net assimilation rates. After weighing, replicate material was bulked, ground, and bottled, and subsequently the leaf samples were analysed for the following ash constituents: potassium, sodium, calcium, and phosphorus, i.e. the variables in the nutrients supplied. Further analyses undertaken on the other plant organs, and the whole of the harvest data, are irrelevant to the present purpose, which is confined to the elucidation of the data for green leaf water content. The complete data of the experiment are given by Shih (1936).

#### EXPERIMENTAL RESULTS

The water-content data are presented in Table III. The most important eighteen treatments, those involving the series A, B, and C may be entered in a symmetrical table and thus conveniently submitted to an analysis of variance. The mean squares derived from such an analysis of the water content including all three sampling periods, for both leaf and stem fractions, are given in Table IV.

As regards the leaf data, it is seen that the main effects of the three nutrient factors and of age are all large, though never significantly greater than all their first order interactions. Potassium supply and the Na/Ca ratio show particularly large effects. The following generalizations may therefore be stated:

TABLE III

*Water Content (per cent. Dry Weight)*

Treatment.	Sample 1.		Sample 2.		Sample 3.	
	Leaf.	Stem.	Leaf.	Stem.	Leaf.	Stem.
HAK <sub>1</sub>	644	987	563	773	486	482
HAK <sub>3</sub>	694	977	645	835	658	929
HAK <sub>5</sub>	919	1,030	850	859	872	1,116
HBK <sub>1</sub>	628	994	582	822	482	469
HBK <sub>3</sub>	651	1,000	608	938	649	890
HBK <sub>5</sub>	782	1,039	817	915	866	1,194
HCK <sub>1</sub>	599	991	520	749	421	421
HCK <sub>3</sub>	541	954	458	739	487	703
HCK <sub>5</sub>	599	832	619	853	637	914
LAK <sub>1</sub>	650	927	552	769	461	497
LAK <sub>3</sub>	612	982	579	794	517	706
LAK <sub>5</sub>	830	994	805	856	834	1,057
LBK <sub>1</sub>	660	921	566	781	482	592
LBK <sub>3</sub>	610	945	561	793	487	622
LBK <sub>5</sub>	669	954	669	877	613	865
LCK <sub>1</sub>	601	918	513	751	430	505
LCK <sub>3</sub>	526	939	473	662	411	539
LCK <sub>5</sub>	563	923	523	696	519	727
HDK <sub>5</sub>	729	1,041	716	919	741	1,099
LDK <sub>5</sub>	636	966	592	825	563	783
HEK <sub>5</sub>	744	993	761	961	834	1,173
LEK <sub>5</sub>	705	1,008	666	858	627	871

TABLE IV

*Analysis of Variance, Water Content*

	Degrees of freedom.	Mean square.	
		(1) Leaf data.	(2) Stem data.
K	2	166,213	156,167
P	1	45,125	60,870
Na/Ca	2	109,743	58,335
Age	2	30,223	243,102
K × P	2	12,283	14,338
K × Na/Ca	4	20,358	8,839
K × Age	4	10,746	101,812
P × Na/Ca	2	2,556	4,031
P × Age	2	3,453	7,362
Na/Ca × Age	4	313	6,494
K × P × Na/Ca	4	2,518	1,940
K × P × Age	4	965	14,213
K × Na/Ca × Age	8	196	1,878
P × Na/Ca × Age	4	703	1,054
Error (K × P × Na/Ca × Age)	8	445	2,876

(1) with the types of nutrient solutions under examination, *increasing deficiency of potassium leads in general to progressively greater succulence*, but this effect is not so uniform as to override statistically the interaction effects of phosphorus level with potassium level; (2) *there is usually a decrease of succulence in passing from series A to C (high sodium and low calcium to no sodium and high calcium)*, but this effect is not significantly greater than are the interaction effects of NA/Ca ratio with potassium level; (3) *the small reduction ( $\frac{1}{3}$ ) of phosphorus level from series H to L leads in general to a lowering of water content*, but the difference is not sufficiently consistent among the series to obscure the effect of any of the first-order interactions involving phosphorus; and (4) *the general age effect from Sample 1-3 consists in falling succulence*, but this effect again is significantly greater than only one of its first-order interaction effects, i.e. the interaction of the sodium-calcium ratio with age.

Of the first-order interactions, that between potassium and phosphorus is statistically greater than its second-order interaction with age, but not than the second-order interaction with the sodium-calcium ratio. The interaction between potassium level and the sodium-calcium ratio is larger, and is not so dependent on either phosphorus level or time of sampling. This interaction may therefore be approximately defined, within the limits of the experimental treatments and sampling times, in the following terms: *water content increases markedly with potassium deficiency at the high sodium levels, and less markedly in nutrients containing medium levels of sodium together with calcium, while at high calcium levels there is a minimum of succulence at about the K<sub>3</sub> level*. The third nutrient interaction, that of phosphorus and the sodium-calcium ratio, is very largely dependent on the potassium level maintained.

Of the first-order interactions between nutrients and age, that of the sodium-calcium ratio is completely negligible. The potassium interaction on age is significantly greater than either of its higher order effects and may therefore be stated in general terms: *during the growth of the plants water content falls rapidly in the series receiving high potassium, much less rapidly at the medium potassium level, and remains high and approximately constant in series with great deficiency*. The remaining age interaction, that with phosphorus, clearly exceeds the error, but does not appear to be significantly greater than either of its second-order interactions; nevertheless it may probably be adequately defined, since none of the three second-order interactions involving age is much greater than the third-order interaction. This latter, with eight degrees of freedom, is taken as the estimate of error, but a better estimate would probably include also the sixteen degrees contributed by the three second-order interactions involving age. The fourth second-order interaction, involving nutrient conditions alone, is clearly real whichever estimate of error be used. The phosphorus-age interaction may therefore be stated: *the decrease in water content ascribable to reduction in phosphorus supply increases with age of plants from sample 1-3*.

To be adequate all other possible statements must take account of the

three nutrient variables, and therefore involve a complex interaction between all four variable elements of the experiment. *The decrease in water content resulting from a reduction in phosphorus level is almost negligible everywhere at high potassium levels; the maximal decrease occurs at medium potassium levels in the presence of high sodium (series A), but at very low potassium levels when calcium level is high (series C). Maximum variability in the difference of water content between the high and low phosphorus levels is found at the lowest potassium level, the difference due to level of phosphorus supply being particularly great in the series containing both sodium and calcium, i.e. BK5.*

Bearing this complex relationship in mind, the two remaining first-order interactions may now be defined in simpler, though less accurate, terms: (1) *the decrease in succulence associated with reduced phosphorus supply is negligible at high potassium levels, but increases markedly as potassium supply is lowered; and (2) the differences in succulence associated with varying phosphorus supply are least at high calcium levels (series C), and greatest in series B containing both sodium and calcium.*

Finally, there remain to be considered the four treatments, not included in the analysis of variance, receiving extra sodium in addition to the other nutrients (series D and E). A comparison between treatments DK5 and CK5, which differ in sodium level alone, shows that the effect of the sodium has been greatly to increase succulence in all the samples taken. This increase is much more pronounced at the higher phosphorus level than the lower. On the other hand, a comparison between treatments EK5 and BK5, both of which contain considerable sodium though the former receives twice as much as the latter, reveals only a small effect of the extra sodium. At the higher phosphorus level the additional sodium has resulted in a slight decrease of succulence, while at the lower the effect is a small increase, probably real.

The empirical results established above for leaves are confirmed by the data for stems. Both the main effects and interactions described for the leaf apply with very little modification to the stem fraction also. The chief difference is a much more rapid decrease of succulence with age in the stem (see Table IV), an effect which is more marked in some treatments than others, so that some striking interactions are noted, in particular, potassium  $\times$  age and potassium  $\times$  phosphorus  $\times$  age. Probably also the higher 'error' in stems reflects merely an increased third-order interaction. But as stated, apart from the relative magnitudes of the effects, responses to changes in nutrient supply are everywhere similar in leaf and stem.

The present results confirm previous data obtained at this Institute with high sodium supply (Gregory and Richards, 1929; Richards, 1938), in so far as data from single leaf samples as formerly used may be directly compared with samples of the whole leaf surface as used in this experiment. Further, these results elucidate the discrepancies in the findings of different investigators which were discussed in the introduction; they indicate that in assessing the effects of nutrient conditions involving potassium deficiency very large



effects, both direct and by interaction with potassium, due to the other ions present, must be taken into account. Until a great deal more is known of the primary and secondary effects of the other ions, and due regard is paid to them, it is quite useless to attempt any statement of general validity as to the effect of potassium supply; all such attempts must be regarded as premature.

In the second part of this paper the data of leaf water content presented above will be analysed in relation to the internal contents of potassium, sodium, calcium, and phosphorus. The analysis reveals strikingly the primary importance of internal concentrations of elements other than potassium in the determination of water content. Since the internal contents of at least some of these are themselves largely dependent on the level of potassium supply, or correlated with internal potassium content, clearly the study of the latter alone in relation to water content may be highly misleading.

### SUMMARY

1. Views expressed in the literature concerning the nature of the relationship between potassium supply and succulence are criticized on the ground that they ignore the complexity of the relationship actually found. The overwhelming importance of the general constitution of the nutrient solution is stressed.

2. An experiment is described in which barley was grown with ten basal nutrient types and at three potassium levels; in all twenty-two manurial combinations were used (see Tables I and II). The basal nutrients consisted of five different combinations of sodium and calcium, together with two levels of phosphorus. Three samples were taken during vegetative growth, and the data of the water content of the leaf and stem fractions are presented (Table III).

3. The differences in water content induced by treatments are examined statistically and important significant interaction effects are found between potassium level and the levels of the other variable elements of the experiment, thus accounting for conflicting statements in the literature. The effects on succulence established statistically are stated in the paper in italics. The following are the most important effects of potassium level: (i) *Increasing deficiency* of potassium leads in general to progressively *greater* succulence. (ii) Water content increases markedly with potassium deficiency at the high sodium level, less markedly at medium sodium level in presence of medium calcium supply, while at high calcium level there is a minimum of succulence at the intermediate potassium level.

4. The following effects of phosphorus and its interaction with potassium and other kations are established: (i) The reduction of phosphorus level to one-fifth of the standard leads in general to a reduction in water content. (ii) The decrease in water content resulting from reduction in phosphorus supply is negligible at high potassium level, but increases markedly as the

potassium level is lowered. (iii) The differences in succulence associated with varying phosphorus supply are least at high calcium level and greatest where both sodium and calcium are present at medium level.

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# A Study of Nut Grass (*Cyperus rotundus* L.) in the Cotton Soil of the Gezira

## I. The Maintenance of Life in the Tuber

BY

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With two Figures in the Text

### INTRODUCTION

*CYPERUS ROTUNDUS* L. is a pest of increasing importance in the cotton area of the Gezira, and the following investigations were undertaken as a preliminary to its eradication.

Work on this plant has been divided into two parts, (i) a study of the maintenance of life in the tuber, (ii) the perpetuation of the plant by means of seed. The first part of the work will be considered in this paper, where it will be shown that complete control can be obtained if the tuber roots are cut during the dry season at a depth below that of the lowest tuber, and the tubers left for a dry period of not less than one month.

Of the more obvious means of control, neither weeding of the land nor insect and fungal pests appear to be of any great value. With regard to the former, Burns (1921-2) has stated that frequent cutting of the aerial parts will finally cause exhaustion and death of the tubers.

In an attempt to demonstrate this effect of cutting, eight 4-gallon petrol tins were filled with fine soil and four tubers sown in each on September 23, 1937. In four tins the aerial growth of the tubers was cut every three or four days at ground-level, while in the remaining four the tubers were allowed to grow normally. The last cut was made on October 31, 1937, and the tubers washed out on October 9, 1937, after cutting down one side of the tins. Diagrams were made of the position and growth of each tuber. It was noticed that extensive roots were present in all cases, though the development was greatest in the tins where the aerial growth was not cut. Formation of new tubers had proceeded rapidly in the control tins; in no case did a new tuber develop on the treated plants. The experiment was not continued sufficiently long to determine if the tubers of the cut plants would eventually die. It would appear that it is necessary for a tuber to establish its aerial growth before it can develop new rhizomes and tubers. Cutting is thus a method of controlling this pest but is obviously too long and laborious to be a useful method of eradication on a large scale.

The tubers of this weed in the Gezira are attacked by a Coccid, but the attacks do not appear to exercise much control of the weed.

During excavation a proportion of the tubers in the top 3 in. depth of soil, and very occasionally in the 3–6 in. depth was found to be soft and shredded inside. Thirty soft tubers collected from a low-lying piece of land were sown, but none germinated though they appeared to be sound externally. The tubers were then washed in sterilizing fluid for four minutes and washed three times in sterile water. Each tuber was then divided across the centre and a piece of the central portion of each tuber was placed on an asparagin agar plate. Practically all the tubers showed internally a dark outer ring and the central portion varied in colour from light brown to black; a few tubers were pulpy and rotted in the centre with only the vascular strands remaining. Four days later 25 of the 30 plates showed only a mixture of a fungus and a bacterium. By the kindness of the Director of the Imperial Mycological Institute the fungus has been identified as *Cylindrocarpon didymum* (Harting) Wollenw., a soil organism attacking dead roots and tubers. It would seem that attack by this fungus is the normal lot of tubers dying in the soil, for the fungus appears to be only very slightly parasitic.

Three diseases of the aerial portions of the plant have been found and the causal fungi have again been identified by the Imperial Mycological Institute. One, a brown spot of the leaf, is due to *Alternaria tenuissima* (Fr.) Wiltshire group, and another, causing a black stripe on the leaf, is caused by a *Physotherma* sp. The third is an attack of the crown at ground-level and the fungus appears to be a species of *Corticium*. As would be expected, these three diseases are most prevalent during the rainy season, but no evidence was found that they could kill the plant or exercise any obvious control on the spread of the weed.

Both Ranade and Burns (1925) and Smith and Fick (1937) show that repeated cultivation of the land will eventually kill the weed. Repeated and intensive cultivation of the Gezira cotton land is not economically possible, so this work was begun in May 1937 to determine the mode of perpetuation of this weed during eight months of hot, dry weather, in six of which no rain normally falls. From this information it was hoped that a swift and effective means of eradication could be devised.

#### MORPHOLOGY OF *CYPERUS ROTUNDUS* L. IN THE GEZIRA

The morphology of this plant in America and India has already been described (Ranade and Burns, 1937, and Smith and Fick, 1937), and from general observations there is no reason to believe that in the Anglo-Egyptian Sudan (hereafter referred to as the Sudan) it differs from its countertype in these two countries. In essentials, as shown in Fig. 1, the plant consists of an aerial and underground structure. The underground portion of the plant is made up of roots and tubers, the latter connected by rhizomes. From the tubers are developed extensive roots and the aerial portion of the plant, the

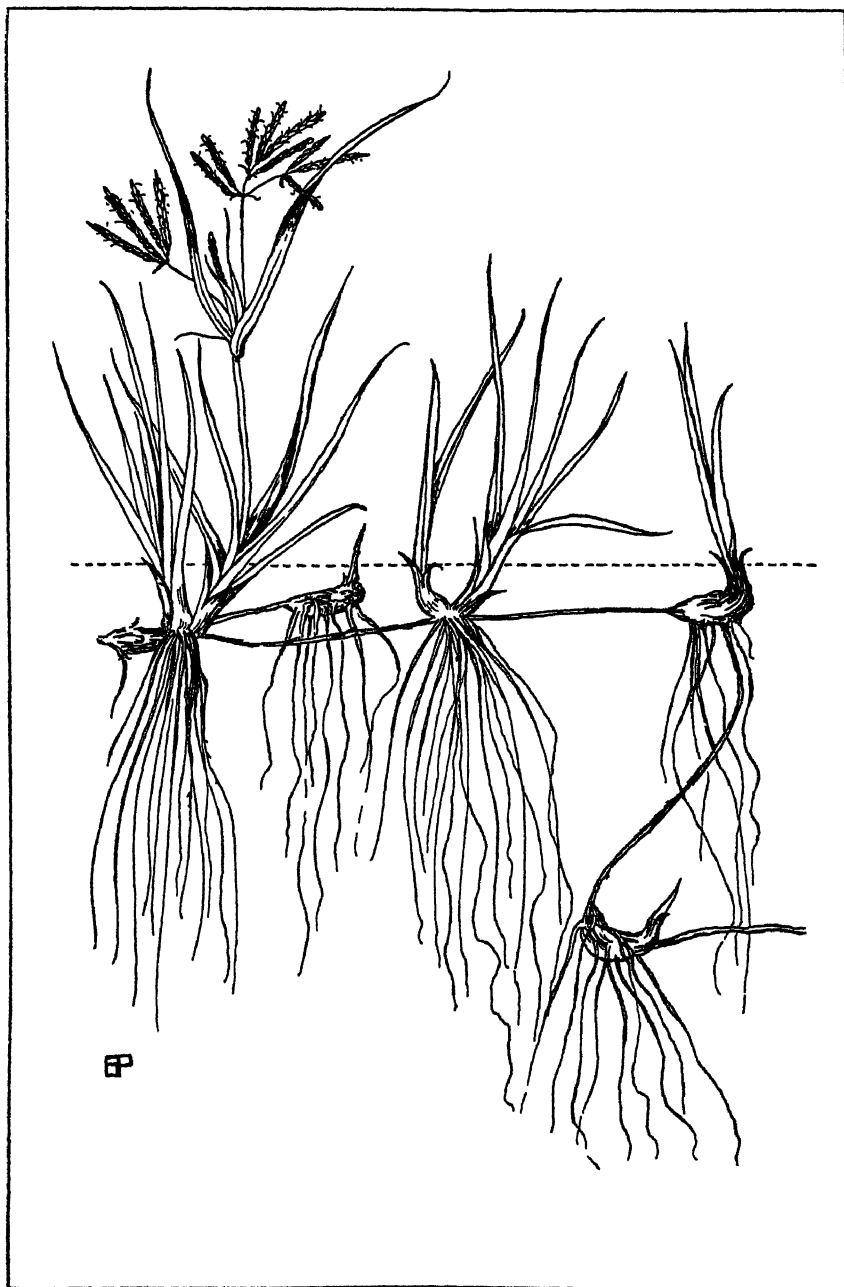


FIG. 1. Drawing of a young plant of *Cyperus rotundus* L. showing aerial growth, tubers, rhizomes, and roots (c.  $\frac{2}{3}$  nat. size). The dotted horizontal line indicates ground-level. Drawn by Miss E. M. Parker.

latter consisting of a rosette of linear leaves from which grow the umbel-bearing flowering stalks typical of the genus; those on the cultivated land of the Gezira Research Farm are not usually higher than about 15 in. The tubers when young are white, but with increasing age their outer covering turns brown and finally black. They seldom exceed 1 in. in length and  $\frac{1}{2}$  in. in width, and consist of nodes and internodes with buds and scale leaves at the nodes. Apical dominance has been demonstrated by Smith and Fick (1937). An underground tuber on germination can give rise to a further rhizome and tuber or produce a rhizome growing towards the surface which eventually produces the aerial growth, or it may do both. At the junction of this upward-growing rhizome with the leaves which occurs below the soil surface, an enlargement will develop which has been called a 'basal bulb' by Ranade and Burns (1937); this resembles a tuber, contains storage material, and can produce further rhizomes. No evidence could be found that the rhizomes connecting two tubers could give rise to new growth. In an experiment where freshly excavated rhizomes, in one case with both tubers, in another with one tuber removed, and a third with both tubers removed, were sown immediately in damp soil, no germination occurred at any of the nodes of the rhizomes, though 48 of the total of 63 tubers produced aerial growth.

#### CLIMATE AND SOIL OF THE GEZIRA

It will be shown below that maintenance of life in the tuber is dependent on the ability of the tuber to resist drought by means of a deep root system tapping a moist subsoil. Before considering the evidence in detail the following particulars of climate and soil may be noted.

The rainfall in the Gezira plain is so distributed that there are normally nearly six months of dry weather. The heavier rains fall from mid-July to mid-October, as is shown in Table I.

The cotton soil of the Gezira (Joseph, 1925) is a heavy clay, strongly alkaline (pH up to 9.4), of low permeability to water and of low content of nitrogen and humus. The following figures show the moisture content of the soil on fallow plots of the Gezira Research Farm after they have received no water for eight months, thus representing the longest possible time that the land is likely to be without water under the climatic conditions of the Gezira.

It will be noticed that below a depth of 24 in. there is a moisture content of 20 per cent. or over, and that from 0 to 12 in. the content varies from 7.4 to 10.8 per cent.

#### DISTRIBUTION OF THE TUBERS IN THE SOIL

Pits, 1 metre square, were dug in different parts of the Gezira Research Farm and in other localities. The soil of each 3 in. depth was carefully removed and washed through a sieve to retain all tubers. The tubers were

TABLE I

*Meteorological Averages. Gezira Research Farm (1919-38)*

Month.	Max. temp. (° C.).	Rel. humidity (%)		Rainfall (mm.).
		8 a.m.		
January . . . . .	33·8	36	—	
February . . . . .	35·1	25	—	
March . . . . .	38·5	20	—	
April . . . . .	41·1	18	2·7	
May . . . . .	41·0	31	10·3	
June . . . . .	39·5	46	33·7	
July . . . . .	35·4	68	132·4	
August . . . . .	33·5	76	146·2	
September . . . . .	35·7	69	62·4	
October . . . . .	38·6	48	10·7	
November . . . . .	36·8	33	1·3	
December . . . . .	34·5	37		

TABLE II

*Moisture Content (per cent.) of Soil after Dry Fallow*

Depth (in.).		Depth (in.).		Depth (in.).	
0-6	7·4	24-30	20·1	48-54	23·6
6-12	10·8	30-36	21·3	54-60	23·4
12-18	15·6	36-42	22·9	60-66	22·2
18-24	17·5	42-48	23·6	66-72	21·6

TABLE III

*Distribution of Tubers at Different Levels as Percentage of Total Tubers*

- (a) 9 pits: Gezira Research Farm: June-July 1937  
 (b) 2 pits: low-lying, water-logged land: Aug. 1937.  
 (c) 2 pits: cultivated land adjacent to (b): Aug. 1937.  
 (d) 6 pits: Gezira Research Farm: Jan. 1938.  
 (e) 6 pits: River Silt: Jan. 1938.

Depth (in.).	(a)	(b)	(c)	(d)	(e)
0-3	59·2	75·8	75·4	30·7	16·4
3-6	31·7	22·5	22·3	56·3	37·0
6-9	8·2	1·6	1·7	10·25	23·1
9-12	0·9	—	0·6	2·70	13·7
12-15	—	—	—	0·05	6·9
15-18	—	—	—	—	2·3
18-21	—	—	—	—	0·5
21-24	—	—	—	—	0·1
24-27	—	—	—	—	—

counted and divided into those that had already produced aerial growth and those apparently dormant. Table III shows the percentage distribution of the total tubers for each 3 in. depth for different localities.

From the table it is seen, column (a), that on the Research Farm the bulk of the tubers occurs in the first 6 in. depth and that no tubers are found below 12 in. Column (d) shows, however, that some may occur as low as



15 in. Other pits dug on the Research Farm during the course of the season showed that though in the great majority of cases the tubers did not go deeper than 12 in., penetration to 15 in. or even 18 in. was very occasionally found.

It was felt desirable to determine if in water-logged land the tubers penetrated deeper than in land of a more permeable nature alongside. Table III, columns (b) and (c), show the results obtained. Greater penetration is found in the more permeable soil and this is confirmed by the data of columns (d) and (e), where a comparison is made of the distribution of the tubers in the Gezira soil and river silt.

In an attempt to induce the tubers to penetrate to the lower layers of the soil, three infested plots were flooded for three weeks in September, three for a similar period in October, and three in November; three unflooded plots acted as control. Moisture contents were determined on all plots seven days after the end of the flooding period. Table IV gives the average percentage moisture content obtained from four estimations at each level in each plot.

TABLE IV  
*Soil Moistures (per cent. of Dry Soil)*

Depth (in.).	September flooded.		October flooded.		November flooded.*	
	Control plot.	Mean of flooded plots.	Control plot.	Mean of flooded plots.	Control plot.	Mean of flooded plots.
0-6	24	39	20	36	8	28
6-12	23	36	24	36	14	34
12-18	22	34	24	34	19	33
18-24	21	33	23	34	20	32
24-30	21	31	22	33	20	29
30-36	21	30	22	33	21	27

\* Samples taken fourteen days after the end of the flooding period. It is probable that only the first 6 in. were affected by flooding.

Variation in the moisture content of the control plots was expected, the degree depending on how soon after a rainfall the samples were taken. Tubers were excavated in the third week of the following April, six plots per treatment (i.e. two pits per plot) being dug. Table V shows the percentage distribution of the tubers per 3 in. depth for each treatment.

No increase in depth of penetration of the tubers was found in any of the flooded plots. The flooded plots do appear, however, to contain more tubers in the 3-12 in. level depth than the controls. This is most obvious in the September flooded plots, less obvious in the October flooded plots, while in those of November the distribution is practically as the controls. It is possible that the higher humidity prevailing during September caused a less degree of evaporation of the water at the end of the flooding period, and there would therefore be greater opportunity for continued growth in the September flooded plots than in the other two.

An examination of the data given in the tables indicates that where water is not limiting during the growth period, as occurred in all the pits examined, some other factor must be inhibiting the further penetration of the tubers in the soil. The least depth of penetration is obtained in waterlogged soil and the greatest in river silt (Table III, *b* and *c*). Lack of aeration is a

TABLE V  
*Distribution at Different Levels of Tubers after Flooding*

Depth (in.).	Control plots.	September flooded plots.	October flooded plots.	November flooded plots.
0-3	49.3	33.6	38.8	52.2
3-6	30.9	43.2	42.4	33.1
6-9	15.5	17.6	15.2	12.1
9-12	2.3	4.8	3.3	2.4
12-15	0.5	0.6	0.14	0.13
15-18	* 2 tubers	* 5 tubers	* 1 tuber	—

\* Occurred in only one of the six plots.

prominent characteristic of waterlogged soil and would be absent from the porous river silt. It is conceivable therefore that where moisture is not limiting sufficiency of aeration is the factor deciding the depth of penetration of the tubers. Great differences exist between the texture of the waterlogged Gezira soil and river silt and a close correlation occurs between lack of aeration in the soil and its physical compactness. It is impossible to be certain that the non-penetration of the tubers may not in part be due to inability of the rhizomes to penetrate the subsoil, though it is penetrated with ease by the tuber roots.

For further light on this aspect, a set of five petrol tins  $9\frac{1}{2}$  in.  $\times$   $9\frac{1}{2}$  in.  $\times$   $13\frac{1}{2}$  in. was filled with loose soil and three fresh tubers sown in each (treatment A). A further set of five tins contained soil that was puddled half-way up the tin and the remainder of the tin filled with loose soil; three fresh tubers were then sown in each tin (treatment B). A final set contained soil puddled to the surface except for 2 in. of loose soil at the top in which three fresh tubers were sown (treatment C). The soil of the tins received periodic waterings sufficient to ensure growth of the tubers. Table VI gives details of the aerial growth.

On November 6, 1937, the tins were cut down one side and the rhizomes, tubers and roots washed out and diagrams made of their positions in the tin.

In treatment A roots, rhizomes and tubers had penetrated to the bottom of the tins. In both treatments B and C roots were distributed through the puddled soil, but the rhizomes and tubers did not penetrate lower than the junction of the loose and puddled soil. Since the puddled soil was moist lack of moisture could hardly be a deciding factor, nor is it likely to be the texture of the soil since the roots, a finer structure, were able to penetrate it easily. It seems conceivable therefore that aeration conditions in the puddled soil

prevented its penetration by the rhizomes. If this is so it suggests that there is a distinct air/moisture relation which must be present in the soil before the rhizomes will be stimulated to penetration.

Table VI shows that in this experiment the extent of aerial growth closely followed the ability of the rhizomes and tubers to penetrate the lower layers of the soil in the tins.

TABLE VI  
*Effect of Puddled Soil on Development*

Tubers sown 18/9/37, aerial growth examined 28-30/10/37.

No. of tin.	Total flowering stems.	Average height (cm.) flowering stems.	Total No. of spikes.	Average length (cm.) of spike.	Aerial dry wt. (gm.).
A					
1	10	21.5	1,151	1.7	21.00
2	21	26.8	1,180	1.3	18.40
3	10	31.5	889	1.6	14.55
4	6	13.8	387	1.3	13.38
5	8	22.6	951	1.8	14.90
Mean	11	23.2	912	1.5	16.44
B					
1	8	23.6	616	1.7	7.45
2	6	13.6	218	1.5	7.45
3	11	21.6	499	1.6	8.05
4	4	20.0	378	2.0	7.45
5	1	26.0	119	1.4	4.35
Mean	6	20.9	366	1.6	7.10
C					
1	8	13.6	305	1.4	4.31
2	—	—	—	—	1.70
3	5	12.5	187	1.3	2.00
4	3	10.5	116	1.3	1.95
5	8	21.5	175	1.8	3.72
Mean	5	14.5	196	1.5	2.73

#### THE ROOT SYSTEM OF *CYPERUS ROTUNDUS* L. IN THE GEZIRA

It was evident in the early stages of this work that the plant possessed in the field an extensive and deep root system. This was confirmed when on the Gezira Research Farm a large pit was dug in an infested area to a depth of 5 ft., and the tuber roots washed out on the four sides of the pit. Table VII shows the results obtained.

No tuber was found below 15 in. depth and the bulk of them were concentrated in the first 6 in.

Fig. 2 is a photograph of the arrangement *in situ* of tubers and roots of plants washed from the side of a deep pit. It will be seen that the roots extend to a depth of 54 in. while the tubers were not found below 12 in. It is not possible in the photograph to observe the extreme fineness of the

lateral roots at the lower depths, but these roots must be of considerable importance in maintaining a water-supply for the tubers in the drier layers of the soil.

#### DORMANCY OF THE TUBERS

All the non-germinated tubers from the pits described in Table III column (a) were immediately sown in river silt in small dishes. Table VIII gives the results from the nine pits on the Gezira Research Farm.

Of the total 264 tubers which did not germinate when sown: 56 were broken in excavation; 89 were small and immature; 5 were bored, presumably by insects; 26 were shrivelled and diseased; 88 showed no apparent reason for non-germination. In the last case it is possible that damage to the root structure of the tubers (see later) was responsible.

The following experiment was carried out to determine the soil moisture necessary for the germination of the tubers. Sifted soil, heated to 150° C. and placed in weighed tins, was moistened with various quantities of water. Three fresh tubers were sown in each tin, and a lid, pierced with a single small hole was sealed on. There were five replicates of each moisture content, giving a total of 35 tins. The results are given in Table IX.

The soil of the 50 per cent. moisture tins had a putrefying smell and the soil with 60 per cent. moisture was of slate blue colour below the surface and had a similar smell; in both cases the soil was liquid in consistency. From this experiment it would appear that at least 30 per

#### SEID GRASS *ICITEPUS RETUNDUS* L

DEPTH OF PENETRATION OF TUBER ROOTS



FIG. 2. Showing penetration of tuber roots to a depth of 54 in.

cent. of soil moisture is necessary for

adequate germination, and that with excessive moisture aeration is likely to be the deciding factor. The effect of the putrefaction on the germination of the tubers is of course unknown. A further experiment in which the tubers were immersed for 14, 21, and 28 days in soil containing 50 per cent. and

TABLE VII  
*Depth of Root System at G.R.F.*

	Side of pit.	Greatest depth (in.) of tuber roots.	Average.
East	. . . .	43—45—44—42	43.5
West	. . . .	54—52—47—44	49.3
South	. . . .	41—40—43—45	44.7
North	. . . .	46—43—44—47	45.0

TABLE VIII  
*Viability of Excavated Tubers*

Depth (in.).	Total tubers.	Germination (%).
0-3	1,257	84.9
3-6	1,100	94.1
6-9	289	96.5
9-12	34	97.1

TABLE IX  
*Effect of Soil Moisture on Germination of Tubers*

Sown 6/8/37. Examined 14/8/37.

Moisture content (%).	No. of buds germinated on 15 tubers.	Root develop- ment.
11.4	Nil	—
21.0	3	Slight
29.9	21	Heavy
36.0	22	Heavy
40.5	19	Heavy
50.7	5*	Nil
61.4	Nil	Nil

\* These occurred in *one* tuber lying partially exposed on surface of soil.

60 per cent. of moisture showed that these conditions induced a state of dormancy but did not kill the tubers. After the period of immersion all tubers were sown in river silt, and germination occurred three days after sowing.

An experiment similar to the former was carried out, but in this case all roots and rhizomes were carefully removed from the freshly excavated tubers which were immediately sown in soil of different moisture contents, the soil being pressed round the tubers to ensure intimate contact. The tubers were tested for viability after a period of three and five weeks. Table X shows the results obtained.

It seems clear that 20 per cent. soil moisture is sufficient to maintain life in the tuber for long periods, but that tubers without roots mostly die within five weeks at moisture contents below 16 per cent. Further information on this point was given by an experiment in which forty pots, diam. 16 cm., were filled with sifted Gezira soil and sown on October 27, 1938, with three

TABLE X  
*Effect of Soil Moisture on Death of Tubers*

Soil moisture (%).	3 weeks Interval No. viable out of total of 9.	5 weeks Interval No. viable out of total of 6.
5	0	1?
11	1	0
16	7	1
20	8	4
22	8	6

TABLE XI  
*Soil Moisture Content (per cent.) at Cessation of Growth*

Pot No.	Top.	Middle.	Bottom.
6	11.0	16.2	15.3
20	16.7	18.2	17.9
39	14.3	17.9	20.2
38	17.1	19.1	17.6
10	16.9	17.8	19.0
17	13.1	16.4	15.8
12	17.7	22.0	21.1
29	20.7	21.8	20.8
24	18.8	19.9	19.1
30	15.2	16.0	16.3
Average	16.2	18.5	18.3

freshly excavated tubers at a depth of 5 cm. The pots were watered from below until November 7, when watering ceased and records of aerial growth were begun. Moisture contents of the soil in ten pots were determined as soon as growth ceased. Table XI shows the results obtained.

At intervals of 3, 5, 7, 9, and 10 days from the date of cessation of growth the moisture content of the soil was determined in the remaining pots, and the tubers resown and watered to ascertain if they were still viable. The results are given in Table XII.

It is clear that wilting of the aerial growth of tubers with an established root system will occur when the roots lie in a soil whose moisture content is below 20 per cent., but the maximum mortality of the tubers does not occur until the soil moisture content round the roots falls to 8 per cent.

Experiments have shown that tubers will germinate if enclosed in a saturated atmosphere, and it is noticeable in the field that the greatest production

of aerial growth occurs during periods of high humidity. During the dry months, though the tubers in the ground are still alive, very little aerial growth is seen. That growth does occur indicates that the presence of soil with 20 per cent. moisture round the tuber is not always necessary. The deep root system of the plant enables it to tap soil-levels of which the

TABLE XII  
*Soil Moisture Content ( per cent.) and Death of Tubers*

Interval (days).	No. of pots.	Moisture contents Average percentage			Tubers sown.	Total still viable.
		Top.	Middle.	Bottom.		
3	5	6.8	13.8	14.6	15	15
5	5	6.6	11.2	12.3	15	11
7	7	5.1	7.3	7.9	17	1
9	7	6.0	7.5	8.8	19	1
10	6	5.3	6.6	7.3	18	1

TABLE XIII  
*Depth of Tubers Producing Aerial Growth*

Depth (in.).	(a)	(b)	(c)	(e)
0-3	53.2	57.6	30.6	57.9
3-6	7.2	38.5	31.1	7.3
6-9	—	12.9	—	—
9-12	—	—	—	—

moisture content is not less than 20 per cent. (Tables II and VII), and the anastomizing system of rhizomes provides for the transport of water from these levels to the topmost tuber.

Table XIII shows the percentage of tubers that had already produced aerial growth when the pits referred to on p. 180 (see also Table III) were dug.

The greater proportion of tubers producing aerial growth occur in the topmost soil layers and, except for (b), which occurred in one pit only, no aerial growth is produced by tubers below the 6 in. level. It is difficult to understand why only 50 per cent. of the topmost tubers normally produce aerial growth when the moisture of the soil surrounding them during the rains must for many days be above 30 per cent. Lack of aeration would hardly occur at such shallow depths.

Observations were made on a piece of infested land that about mid-November 1937 had been cultivated to a depth up to 7 in. When examined at the beginning of January 1938 it was noticed that new green aerial growth had appeared sporadically over the land, though it had received no water since the last rainfall at the beginning of November. Examination of 30 of these germinated tubers showed that in all cases except one the tubers lay below the level of the soil disturbed by the cultivator. One explanation might be that the opening of the soil by cultivation had increased aeration and so enabled the tubers to germinate on the water supplied by the deep

root system. Another explanation, suggested by Dr. F. Crowther, is that the topmost tubers, having been severed from the plant, a greater food supply reaches the lower tubers and in consequence they develop aerial growth. Experimental evidence for either explanation is at present lacking.

#### EFFECT OF EXPOSURE ON MORTALITY OF THE TUBERS

Numerous experiments have shown that exposure of freshly excavated tubers to the air and sun causes death after fourteen days. Table XIV shows a typical result. One hundred tubers were used for each treatment; they were sown at the end of the period of exposure and examined twenty-one days later.

TABLE XIV  
*Death Rate of Tubers Exposed to Sun and Air*

Exposure (days).	Germination (%)
0	100
1	93
3	47
5	16
7	7
14	0

These experiments were carried out in July and August with many cloudy days and high humidity. Death is presumably more rapid in drier weather.

In another experiment freshly excavated tubers were buried in soil in boxes so that there were 6 in. of dry soil above the tubers and at least 6 in. of similar soil below. Tubers were buried in two boxes on November 27, 1937, and a light watering given to one of them. On December 23 the tubers were excavated from the two boxes with the result shown in Table XV.

TABLE XV  
*Death Rate of Tubers in Dry Soil*

	No. of Tubers	
	Germinated.	Not germinated.
Box not watered . . .	—	60
Box watered . . .	*10	29

\* Germinated in the soil, but no aerial growth appeared above the soil.

All the ungerminated tubers were then sown in river silt and watered, but no germination of any of them had occurred by January 9, 1938 (i.e. 17 days later). Of 87 fresh tubers sown immediately on excavation 87 germinated.

The effect of heating in an oven was also tested on freshly excavated tubers. After the period of heating the tubers were sown and germination counts were made after an interval of nineteen days. The results are given in Table XVI.

It is evident that exposure to a temperature of 60° C. causes high mortality



among the tubers. This temperature is frequently exceeded at the soil surface, but we have no data as to the temperatures below the surface.

Since the tubers are so easily killed by drought and heat, it is clear that during the dry season they must have some source of moisture which enables them to survive. The following experiment was accordingly undertaken.

TABLE XVI  
*Death Rate of Tubers Exposed to Heat*

100 tubers per treatment.

Exposure (min.).	Germination (%)	
	40° C.	60° C.
0	92	97
30	89	17
60	94	3
120	71	7

Tubers with their roots were washed out on November 30, 1937, kept in water until 8.30 a.m. on December 1, 1937, when 24 of them were supported on bottles of capacity 1 litre. Two tubers were placed in each bottle so that the roots were immersed in a culture solution, the tubers themselves being held permanently free of the water. The bottles were exposed on the roof so that the lower parts of them were shaded but the tubers were fully exposed to the sun. Nineteen tubers were exposed on bottles containing water, but the roots were left *outside* the bottles, and 29 tubers were exposed in a dish *without* water. The experiment was discontinued at 1.0 p.m. on December 10, 1937, and all ungerminated tubers were sown in river silt and examined on December 26, 1937. It was noticed that even during the hottest part of the day some of the tubers with their roots in water showed continually a glistening film of water covering them.

The experiment gave the following results. On December 10, 1937, of the tubers with roots in water, 8 had germinated and produced aerial growth; of the 16 remaining 10 were found to be viable after sowing. Thus out of a total of 24 tubers exposed to the sun for ten days with their roots in water, 18 remained viable. Of the controls, (a) tubers sown immediately on December 12, 1937, showed 30 viable tubers out of a total of 33; (b) of the tubers exposed *over* water with roots outside the bottle, all the 19 tubers were dead at the end of ten days; (c) of the tubers exposed in a dish all the 29 tubers were dead at the end of ten days.

The fact that tubers with their roots in water, subsequently found to be non-viable, remained covered with a film of moisture during the heat of the day indicates that such a condition does not prevent the lethal effect of exposure.

In another experiment fresh tubers were washed out with their roots on November 29, 1937, and transplanted to twelve pots containing river silt, with two tubers in each pot. The roots were in the soil and the tubers exposed to the air and sun on the surface of the soil (treatment A). A similar

set of pots was prepared, but both tubers and roots were on the surface of the soil (treatment B). The pots were watered by standing them in dishes of water. By December 12 14 tubers in the A series had produced aerial growth and two in the B series. On the latter date all non-germinated tubers were sown in river silt. The total germination (including those germinated in the pots) on January 9, 1938, is given in Table XVII.

TABLE XVII

*Death Rate of Tubers with Exposed Roots*

Treatment A.		Treatment B.	
No. of tubers.	No. germinated.	No. of tubers.	No. germinated.
24	17	23	2

The germination of the two tubers in the B series was most probably due to the impossibility of preventing the water rising to the surface of the soil and moistening the *tubers*.

The capacity of tubers to withstand exposure so long as their root system is supplied with moisture was confirmed in the field by an experiment carried out on infested plots that were covered with a thick layer of sorghum stalks during the third week of July 1937. Tubers normally develop below the soil surface, but under this treatment non-germinated tubers were found on the soil surface immediately below the sorghum covering. Three 4-metre squares were marked out on January 5-8, 1938, after the covering had been removed, and about half the number of 'surface' tubers in each square were detached from their soil connexion, and exposed beside the square. The remaining 'surface' tubers were exposed *in situ*. On January 23 (fifteen days after the beginning of this experiment, all tubers were collected and sown to test their viability. Table XVIII shows the results obtained.

TABLE XVIII

*Effect of Severing Roots on Death Rate of Exposed Tubers*

Square No.	Free tubers.			Attached tubers.		
	1	2	3	1	2	3
Total tubers	28	30	40	31	44	31
No. germinated	0	0	0	26	35	23
Percentage germination	0	0	0	83.8	79.5	74.2

Thus out of 98 tubers separated from their roots in the soil none survived fifteen days' exposure; of 106 tubers exposed while attached to their root system 84 (i.e. 79 per cent.) survived.

Tubers lying on the surface which, while under the sorghum covering, had produced aerial growth during the rains, were also collected after fourteen days' exposure and sown. The results are given in Table XIX.

Thus out of a total of 968 tubers exposed on the soil surface which had

previously germinated, 592 (= 61.2 per cent.) remained viable, and were capable of producing further aerial growth when the moisture conditions become favourable.

TABLE XIX  
*Viability of Previously Germinated Tubers*

Square No.	1	2	3
Total tubers . . . .	324	447	197
No. germinated . . . .	205	279	88
Percentage germination . . . .	63.9	66.9	44.6

It seems clear from the above results that tubers, whether exposed on the soil surface or in a soil of low moisture-content, remain alive only if they are able to obtain continuously sufficient moisture from a moist subsoil.

#### EFFECT OF DEEP CULTIVATION OF THE LAND ON MORTALITY OF THE TUBERS

From the results given above it would seem that complete eradication of this weed, apart from plants growing in the rains from seed, should be accomplished if the roots of the tubers could be cut during the dry season, and the tubers then left for a period of not less than fourteen days. It is essential, however, that any instrument used for this purpose should work at a depth below that of the lowest tuber if complete eradication is to be obtained.

Experiments in the field on these lines, using a machine cultivating to a depth of 12 in., were carried out in March 1938 and proved extremely successful. Complete eradication was not, however, obtained owing to the impossibility with the instrument used of maintaining a *constant* depth. Though the most frequent depth was about 12 in., areas occurred where the depth varied between 12 in. and 8 in., owing to the intractability of the soil and the type of machine used. On the areas cultivated to a lesser depth the tubers whose roots were uncut germinated in the following rains. The experiment emphasized the need for considerable overlapping of the tine points of the cultivator. Owing to their 'whippy' nature the rhizomes with attached tubers tended to bend round the tine points, particularly where the soil was very dry and crumbled easily. In consequence the tubers still remained attached to their root system and so survived. It was also demonstrated that in the field tubers enclosed in a lump of soil required nearly a month of dry weather before they died. The results so far obtained by this deep cultivation indicate that a swift and effective method of eradication involving only one operation has been devised. The degree of success achieved would naturally be governed by the type of cultivator used and its ability to work at a *constant* depth in the soil. Though tubers are known to occur in the Gezira soil at depths below 12 in. such occurrence is extremely infrequent. It is therefore felt that cultivation at a depth of 12 in. will cause such a high mortality among the tubers that any that do remain and produce aerial growth can be dealt with in the normal process of weeding the cotton land before the crop is sown.

### SUMMARY

A description of the vegetative characters of *Cyperus rotundus* L. in the Gezira is given. The tubers of this plant do not normally lie deeper than 12 in. in the Gezira cotton soil.

Tubers were exposed to the sun and to oven heat under various conditions. Tubers severed from their roots died after an interval of fourteen days' exposure to the sun.

Deep cultivation of the land during the dry season, to a depth below that of the lowest tuber, thereby severing the tuber roots, provides a rapid and effective method of eradication, and involves only one operation.

### ACKNOWLEDGEMENTS

I have to record my indebtedness to the late Mr. M. A. Bailey, formerly Director of the Agricultural Research Institute, Wad Medani, under whose direction these researches were made. I would also express my thanks to the Manager, Sudan Plantations Syndicate, by whose courtesy the deep cultivation work was carried out.

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## NOTES

### NOTES ON SOME AUSTRALIAN SOOTY MOULDS

*Chaetothyrium Citri* (Arn.) nov. comb.

The occurrence of this fungus in Australia has not been previously recorded. In Victoria, however, pale sooty coverings in which this species forms the dominant constituent occur quite commonly. Collections have been made in coastal districts, where *Alyxia buxifolia* R. Br. is a common host, and also farther inland, on *Bursaria spinosa* Cav.

The hyphae are olive-brown (Ridgway<sup>1</sup>), sparsely or irregularly septate, and devoid of hyphopodia. The ascocarps (av.  $118\mu$  diam.) and pycnidia (av.  $70\mu$  diam.) are sessile and hemispherical; they usually bear a few stiff-pointed bristles which are non-septate and darker in colour than the ordinary hyphae. The asci, which are thick-walled and provided with an apical pore, are 8-spored. The mature ascospores are hyaline or light-olivaceous, elliptical spindle-shape, and divided into cells by (3-7) transverse septa. The measurements of these spores vary from  $15-31\mu$  in length and  $4-7\mu$  in width, but average  $22\mu \times 5\mu$ . The pycnosporos are hyaline or light-olivaceous, unicellular rods, measuring  $5-7\mu \times 2-2.5\mu$ .

In the districts surrounding Melbourne a seasonal spore development has been observed. Late in the spring (November), pycnidia are abundant, but ascocarps are rare and the ascospores are immature. Towards the end of December mature ascospores may be found and a plentiful development of these spores continues until the middle of March. By the end of April, however, ascocarps are rarely found and then these are usually empty. The asexual cycle is slightly in advance of the ascigerous one; and early in February the production of pycnidia begins to decline. These facts tend to confirm Arnaud's opinion<sup>2</sup> (p. 244) that summer conditions are unfavourable to the growth of 'sooty moulds'.

*Taxonomy.* This species was first found on a variety of host plants near Montpellier. Arnaud then described it under the name *Pleosphaeria Citri* nov. nom., but later<sup>3</sup> he transferred it to the genus *Limacinia*. Arnaud identified this fungus with what he considered to be a single species known by the following names: *Limacinia Citri* (Br. et Pass.) Sacc., *Limacinia Penzigi* Sacc., *Limacinia Cameliae* (Catt.) Sacc. Only hyaline ascospores were seen by him, but assuming that he was dealing with immature ascocarps, he attributed a brown colour to the ripe spores.

Woronichin<sup>4</sup> (p. 245) criticized Arnaud's synonymy, which was established on descriptions only of the above-mentioned fungi. He considered Arnaud's fungus to be a distinct species; and he renamed it *Aithaloderma Citri* (Arn.) Woronich.

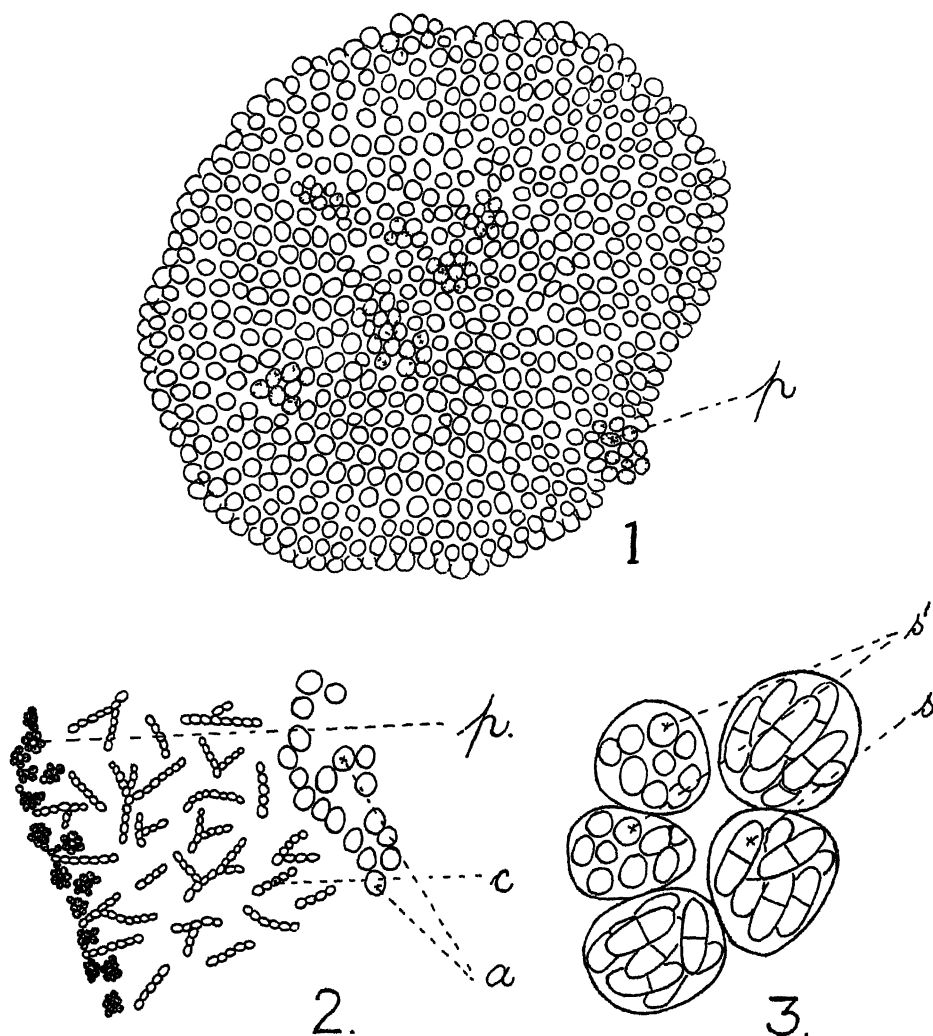
<sup>1</sup> Ridgway, R., 1912: Color Standards and Color Nomenclature. A. Hoen & Company, Baltimore, M.D.

<sup>2</sup> Arnaud, G., 1912: Contribution à l'étude des fumagines, III. Ibid., xii. 1-34.

<sup>3</sup> Arnaud, G., 1910: Contribution à l'étude des fumagines, I. Ann. École nat. d'Agr. Montpellier, 2 sér., ix. 239-77, pl. 1-3.

<sup>4</sup> Woronichin, N. N., 1926: Zur Kenntnis der Morphologie und Systematik der Russtaupilze Transkaukasiens. Ann. Myc., xxiv. 231-64.

Woronichin gave a detailed description of this species from a specimen which he identified with Arnaud's fungus, although it had been previously referred by Briosi and Cavara to *Meliola Penzigi* Sacc.



FIGS. 1-3. *Phycopsis australiensis*. Fig. 1. Surface view of thallus.  $\times 450$ . Fig. 2. Portion of thallus which has been crushed to show asci.  $\times 200$ . Fig. 3. A group of asci.  $\times 1,000$ . p, propagulum; s, ascospore; s', ascospores seen end on; a, asci; c, chain of hyaline cells.

Through the courtesy of Dr. Arnaud I have been able to examine the type-specimen. Woronichin apparently saw no pycnidia, but otherwise his description is accurate, and for the most part I agree with his treatment of this species. *Aithaloderma* Syd., however, should not be considered as a separate genus; it has been distinguished from *Chaetothyrium* Speg. char. emend. Theiss. by unreliable and

inadequate characters (Fisher<sup>1</sup>). The new combination *Chaetothyrium Citri* (Arn.) nov. comb. is therefore established.

*Phycopsis australiensis* n. sp.

Thallo globoso, 50–250 $\mu$  lato, cum seriebus hyalinibus in muco immersis, umbro Saccardinis infecta cum superficie, propagulis sparsis temere per hanc regionem. Quidque propagulum plerumque 16 $\mu$  latum, racemo cellularum fuscarum globoso 4 $\mu$ . Asci rari sepulti in medio thalli corpore cum muris tenuibus, 14–20 $\mu$  altis  $\times$  11–17.5 $\mu$  latis ascosporis hyalinis 1 septatis 11.5–13.5 $\mu \times$  3.5–5 $\mu$ . *Hab.* Foliis *Bursariae spinosae* Cav. Lilydale et Killara, Victoria, reperto.

In an earlier paper (Fisher<sup>2</sup>) record was made of an unidentified species of *Phycopsis*. This fungus has since been found in the perfect state; and its ascospore measurements serve to distinguish it from all described species of the *Atichiaceae*, except *Atichia glomerulosa* (Ach.) Flot. The latter, however, exhibits features characteristic of the genus *Atichia*; the asexual reproductive organs are restricted to particular areas of the thallus, and this, furthermore, is lobed when mature. The fungus under consideration is therefore assigned to a new species, *Phycopsis australiensis*.

The spherical thalli, which vary in size from 50–250 $\mu$ , consist of chains of hyaline cells imbedded in a mucilaginous matrix. The surface of the thallus is coloured with Saccardo's umber (Ridgway); and the propagula are distributed at random throughout this region. Each propagulum measures approximately 16 $\mu$  in diameter and consists of a spherical cluster of brown cells (aver. diam. 4 $\mu$ ). These asexual reproductive organs are developed at all times of the year, but only once has the fungus been found in the ascigerous condition. The asci were imbedded in the tissue of the thallus and restricted to its central part (Fig. 2). They were thin-walled, and their dimensions varied from 14–20 $\mu$  in length and 11–17.5 $\mu$  in width. The hyaline bicellular ascospores measured 11.5–13.5 $\mu \times$  3.5–5 $\mu$ .

*Phycopsis australiensis* was isolated in culture; and an extremely mucilaginous, olivaceous black (Ridgway) growth, developed. On a liquid medium, such as a solution of 5 per cent. honey in water, the fungus extended uniformly over the surface in a thin film. Microscopic examination of such a culture revealed cushions of cells, which may only be distinguished from the thalli occurring on the host, by the larger size (20–30 $\mu$  diam.) of the propagula. In some cultures chains of hyaline cells, which may become elongate and almost filamentous, were also produced.

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**APPARATUS FOR THE PROLONGED EXPOSURE OF PLANT SECTIONS TO CHLORINE GAS.**—In a previous paper,<sup>3</sup> which recorded the results of an investigation into the chemical nature of the cell-membrane of plants, an apparatus was devised for the prolonged exposure of the sections of plant stems to chlorine gas at room temperature. As the work progressed the use of liquid chlorine in a cylinder was found to be cumbersome and inconvenient in

<sup>1</sup> Fisher, E. E., 1939: A Study of Australian 'Sooty Moulds'. *Ann. Bot., N.S.*, iii, 399–426.

<sup>2</sup> Fisher, E. E., 1933: The 'Sooty Moulds' of some Australian Plants. *Proc. Roy. Soc. Victoria, N.S.*, xlv, 171–202.

<sup>3</sup> Wood, F. M., 1926, *Ann. Bot.* xl, 548,



many ways. It was found that if a large apparatus was used so as to have enough chlorine to complete the reaction, the difficulty of so long an exposure to a current of chlorine gas could be avoided.

The apparatus consisted of a cylindrical glass jar with a ground-on glass lid, and with inlets and outlets for the gas closed with stoppers carrying tubes provided

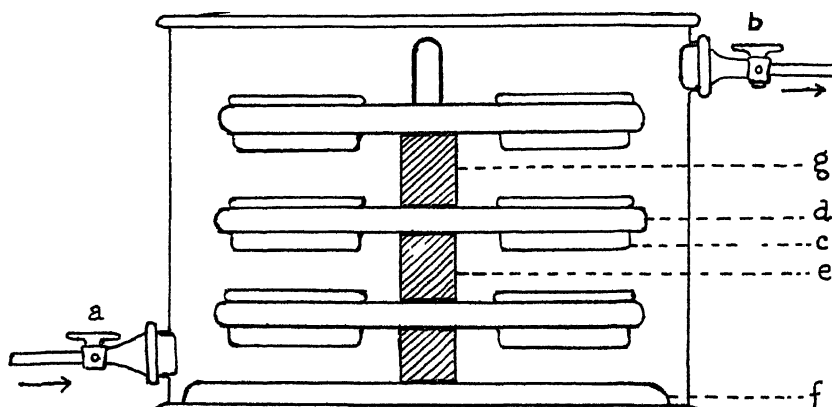


FIG. 1.

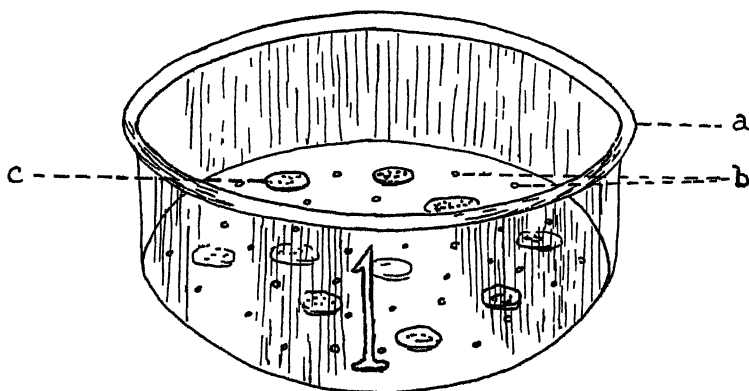


FIG. 2.

with glass taps, Fig. 1, *a* and *b*. The plant sections were placed in numbered glass dishes with perforated glass bottoms, Fig. 1c and Fig. 2. These were held in sockets in circular glass plates *d*, which were mounted on a pillar *e*, supported by a heavy glass base *f*. Several of these plates were used and were separated from one another by glass 'Stops' *g*. The whole of this part of the apparatus stood inside the large vessel, and was easily removable.

Since the gas was chlorine no metal could be used in the apparatus. Glass is also convenient when the apparatus is used with other gases.

After being washed with water the chlorine was passed through the apparatus until the air had been displaced. When the exposure of the sections to the gas had been sufficiently long, the chlorine was removed by suction through a filter-pump. The supporting apparatus for the sections can be removed in its entirety, so that they are washed readily with water *in situ* in the dishes. The glass plates carrying the dishes can be revolved so that the drip of products of chlorination from one tier to the next can be prevented by rotating the discs.

The advantages of this arrangement over that previously designed are that chlorine from a Kipp's apparatus can be used, the worker is less exposed to chlorine fumes, less gas is required, more complete washing of the sections can be secured, and the apparatus is suitable for use with other gases.

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## CORRIGENDUM

N.S., Vol. IV, No. 13, p. 193.

F. W. Andrews: A Study of Nut Grass (*Cyperus rotundus* L.) in the Cotton Soil of the Gezira. I. The Maintenance of Life in the Tuber.

*To the literature cited should be added:*

SMITH, E. V., and MAYTON, E. L., 1937: Nut Grass Eradication Studies. II. The Eradication of Nut Grass (*Cyperus rotundus* L.) by Certain Tillage Treatments. Jour. Amer. Soc. Agron., xxx. 18.



# Chromosome Numbers and the Relationship between Satellites and Nucleoli in *Cassia* and certain other Leguminosae

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With forty-three Figures in the Text

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## I. INTRODUCTION

ALTHOUGH the Leguminosae is recognized as the second largest family of the Dicotyledons, the Compositae alone exceeding it, it is nevertheless a relatively natural group, and it is also one of the most important families from an economic point of view. The seeds of the various beans, peas, ground-nuts, &c., are rich in starch and proteins and form a widespread source of food. Species of *Vicia*, *Trifolium*, *Sesbania*, &c., serve as excellent fodder. Some of the tropical species, such as *Crotalaria*, *Aeschynomene*, yield fibre; quite a number of tropical trees yield useful woods; gums are extracted from *Acacias*, and resins and balsams from *Copaifera*, *Hymenaea*, &c. *Indigofera* and *Genista* yield useful dyes, *Sesbania*, *Trifolium*, *Pisum* serve as an

<sup>1</sup> Part III of thesis accepted for the Ph.D. Degree of the University of London.

excellent green manure, fixing the atmospheric nitrogen in the soil. The family also contains a number of plants of medicinal value, e.g. species of *Acacia*, *Tamarindus*, and *Astragalus*. A number of ornamental trees and shrubs as *Cicer*, *Siliquastrum*, *Cytisus*, *Acacia*, and climbers like *Wistaria*, *Clitoria* also belong to this family.

The family is also interesting in other ways. From *Lathyrus* the first evidence of a definite morphological relation between the nucleolus and the chromosomes was obtained (Latter, 1926). Again, from a study of the various species of *Vicia*, Heitz (1931*b*) first concluded that probably all plants have satellited chromosomes which give rise to nucleoli in telophase. A study of the somatic chromosomes is instructive in various ways. It brings to light not only the numerical variation (polyploidy and aneuploidy) but also indicates the nature of the evolution of the chromosome complements in the various species and allied genera. The size of the chromosomes together with the positions of the spindle-attachment regions which determine the arms of the chromosomes, the secondary constrictions and the satellites indicate the chromosome type in a species. Any changes observed in the positions of these lead to the inference of translocation, fusion, or fragmentation according to the changes involved. Study of the karyotype alterations on these lines in several plant groups such as *Liliaceae* (Delaunay, 1926; Sato, 1936), *Crepidinae* (Babcock *et al.* 1937), and *Allium* (Levan, 1935) has furnished interesting results.

Although the chromosome numbers of many plants of this family have been reported (Kawakami, 1930; Senn, 1938), yet little attention has been paid by investigators to determining the number of SAT-chromosomes in the complement and their relationships to the nucleoli. The usefulness of such a study first came to be recognized when De Mol (1926) pointed out that the number of nucleoli in the cells was a reliable guide to the polyploidy of the plant. Later, Heitz (1931 *a* and *b*) showed that the number of nucleoli formed in telophase nuclei depends upon the number of SAT-chromosomes in the complement. Gates (1938) has shown how the study of satellites and nucleoli can be brought to bear on the phylogeny of the nucleus and a variation in their number and size correlated with allo- or auto-polyploidy. These data, when correlated with the maximum secondary pairing, form a reliable guide to the basic number of the species or genera. Thus it has been pointed out that three species of *Lactuca* ( $2n = 16$ ) investigated by Babcock *et al.* (1937) are probably secondary tetraploids of amphidiploid origin, each having four SAT-chromosomes in the complement. Again, rice, with  $n = 12$  chromosomes, has been shown to have originated through secondary polyploidy from an ancestral condition in which  $n = 5$  (Nandi, 1936). Similarly *Cicer* (Iyengar, 1939), and *Sesbania speciosa* and *S. Sesban* (Jacob, in press) have been shown to be secondary tetraploids evolved from a basic set of four. Senn (1938), in an analysis of the chromosome number relationships in the Leguminosae, finds that the percentage of polyploidy in the family is very low,

only 23 per cent. being polyploids or derived from polyploids. As stated above, *Cicer*, *S. speciosa*, and *S. Sesban*, which were considered to be diploids, have been shown to be secondary tetraploids. There is no doubt that a re-examination of many of the supposed diploids will present a different picture, so that the percentage of polyploidy in the family is bound to go up considerably.

Although many obscurities regarding the origin and organization of the nucleolus had been cleared up by the publication of the classical works of Heitz (1931 *a* and *b*) and McClintock (1934), there are still a number of points which require elucidation. It has been reasonably established that the nucleolar material is derived from the matrix of the chromosomes, but it is not clear if all the chromosomes contribute material towards the organization of the nucleolus. Also the nature of the change is little understood. Again, although McClintock (1934) has shown the presence of a deep staining body at the base of the satellite thread in chromosome VI of the monoploid complement of *Zea mays*, which is responsible for the organization of the nucleolus, it is not clear whether such a body is present in the SAT-chromosomes of all species. Very little is known regarding the exact function of the nucleolus in the economy of the plant cell.

It was with the idea of throwing more light on some of the above problems that the present work was undertaken.

## II. MATERIAL AND METHODS

The following species were investigated.

1. *Cassia auriculata* L. A tall shrub with nearly sessile leaves and large foliaceous stipules; flowers large and showy, bright yellow, in copious corymbose racemes; pods 4-5 in. long with a distinct space between the uniseriate seeds. It is found wild in the Central Provinces, India, and Ceylon, and is often cultivated.

Seeds were sown at the Courtauld Genetical Laboratory, Regent's Park, London, in pots kept in the greenhouse. Two types of plants were present in the culture: (*a*) type I, as described above; (*b*) type II, a single plant which exhibited a more vigorous growth with larger leaves. It did not flower at the same time as the others, but grew to a greater height. This plant perished in the greenhouse during the cold spell in December 1938, before flowering.

2. *C. Tora* L. An annual weed growing into an undershrub with distinctly petioled leaves. Flowers usually in nearly sessile pairs in the axils of leaves with small bright yellow corolla. Pods 6-9 in. with the seeds flattened in the same direction as the pod. Found wild throughout India up to a height of 5,000 ft.

3. *C. javanica* L. A tree reaching a height of about 50 ft. with leaves about 1 ft. long. Flowers in racemes. Pod about a foot long. Found from the Eastern Himalayas to Malaya.



4. *C. grandis* L. Seeds from cultivation in Ceylon.
5. *C. floribunda* Cav. A shrub with three to five pairs of leaflets and yellow flowers. Tropical America.
6. *C. siamea* Lam. A low tree with leaves 6–12 in. long. Corymbs crowded with bright yellow flowers, pod 6–9 in. long, straight and flat. India (Western Peninsula), Malaya, and Siam.
7. *C. corymbosa* Lam. A tropical shrub with leaves having three pairs of leaflets and devoid of stipules. Flowers yellow. Tropical America.
8. *Caesalpinia pulcherrima* Swartz. A shrub or a small tree with a few prickles scattered on the branches. Leaflets sessile. Racemes very broad with round reddish-yellow petals which are distinctly clawed; pod 2–3 in. long and nearly straight. Usually cultivated throughout India.
9. *Poinciana regia* (Boj.) Hook. A tree with leaves about 6–9 in. long and flowers in corymbose racemes; pod 6–8 in. long. Frequently planted in India.
10. *Clitoria ternata* L. A climber with slender downy stem having minute linear stipules. Flower papilionaceous with bright blue standard petals; pod 2–4 in. long. Found in the tropical zone from Himalayas to Ceylon and Burma. Usually cultivated.

I am indebted to Professor R. R. Gates for seeds of 1, 3, 4, and 8, which he obtained from a horticulturist in Kandy, Ceylon; to Miss M. P. Oommen, M.A., Women's College, Trivandrum, for seeds of 2, 6, 9, and 10, and to Mr. C. Robinson, Curator, Chelsea Physic Gardens, London, for root-tips of 5 and 7.

Root-tips were collected from plants grown in pots as well as from seedlings germinated between moist blotting sheets, on bright days between 10.30 and 11 a.m., and fixed in Levitsky's fluid (5 : 5), an evacuating pump being used whenever necessary. The fixed materials were washed from twelve to twenty-four hours in running water and dehydrated by the chloroform method. Sections were cut from 8 to 12  $\mu$  thickness. The slides were stained by the Feulgen-fast green technique described elsewhere (Jacob, in press).

### III. OBSERVATIONS

Table I gives a list of the chromosome numbers observed in the various species of *Cassia*, *Caesalpinia*, *Poinciana*, and *Clitoria*, together with the number of nucleoli formed at telophase. The number of SAT-chromosomes in the complement as well as the attachment of the chromosomes to the nucleolus at prophase were also determined wherever possible. Owing to the smallness of the chromosomes it was not possible to observe the satellited chromosomes in some species of *Cassia* as well as the attachment of these chromosomes to the nucleolus at prophase.

#### 1. *Cassia auriculata* L.

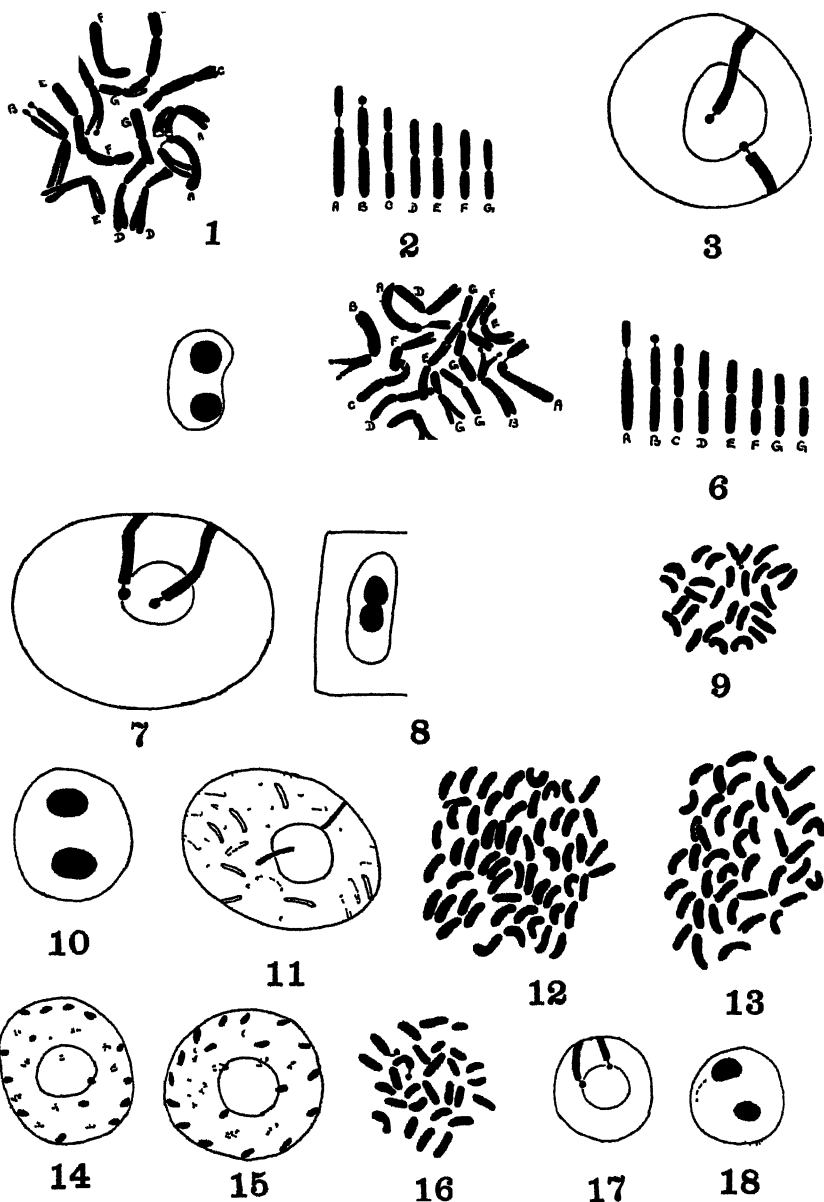
(a) Type I ( $2n = 14$ ). The diploid complement consists of seven pairs of chromosomes of which one pair is satellited. Fig. 1 shows a polar view of the

TABLE I

No.	Name of Species.	2n	No. of SAT-chromosomes in the complement	No. of nucleoli formed at telophase	No. of chromosomes attached to the nucleolus at prophase.	Whether previously reported or not.
1.	<i>Cassia auriculata</i> L.					
	(a) Type I	14	2	2	2	Not reported
	(b) Type II	16	2	2	2	"
2.	<i>C. Tora</i> L.	28	—	2	2	$n = 13$ (Datta, 1933, & Senn, 1938)
3.	<i>C. javanica</i> L.	28	2	2	2	Not reported
4.	<i>C. grandis</i> L.	28	2	2	—	"
5.	<i>C. floribunda</i> Cav.	28	—	2	—	"
6.	<i>C. siamea</i> Lam.	28	4	4	—	"
7.	<i>C. corymbosa</i> Lam.	28	—	4	—	"
8.	<i>Caesalpinia pulcherrima</i> Swartz.	24	4	4	4	$2n = 24$ (Senn, 1938)
9.	<i>Ponciana regia</i> (Boj.) Hook.	28	—	7	—	Not reported
10.	<i>Clitoria ternata</i> L.	16	4	4	4	"

somatic metaphase. Fig. 2 represents the haploid set arranged according to their lengths. The complement consists of one pair of long chromosomes, two pairs of median chromosomes of which the longer pair is satellited, and four pairs of slightly shorter chromosomes. All the chromosomes form a graded series without any wide gaps. The c pair of chromosomes is secondarily constricted, but does not organize nucleoli. Secondarily constricted chromosomes which do not form nucleoli have been reported in other plants such as *Scilla* and *Haworthia* (Sato, 1936); *Narcissus* (Fernandes, 1936), *Sesbania grandiflora* and *S. Sesban* (Jacob, in press). According to Sato (1936), secondary constrictions which do not organize nucleoli may have an origin entirely different from the ordinary constriction or from the satellite and may indicate either a 'trace of translocation or inversion' or crossing-over. Levan (1936) showed in a hybrid between *Allium Cepa* × *A. fistulosum* that new chromosomes were produced directly by crossing-over.

The longest pair (A) in the complement presents a different picture. Here the constriction is drawn out into a long thread which in this species is quite characteristic of this pair. The base of the constriction is slightly swollen, suggesting a satellite. If this contention is true, then this chromosome will represent a satellited chromosome in which the satellited arm became inverted, with the result that the satellite assumed an intercalary position. A case presented elsewhere (Jacob, in press), in which a lateral satellite at the constriction of the chromosomes was attached to the nucleolus, indicates that the satellited arm can be inverted without losing its capacity for organizing the nucleolus. Since this pair does not organize nucleoli it has to be assumed that the nucleolar organizers of these chromosomes were lost. The long-drawn-out constrictions in the present case may be mechanically unsound as they might interfere with the proper orientation and division of the chromosome.



All drawings were made at bench level with the aid of a camera lucida. An achromatic objective N.A. 1.3 was used in conjunction with Zeiss eyepieces K. 25 and 10, giving an approximate magnification of 4,000 and 1,850 diameters respectively. Fig. 43 was drawn with 10, the rest with 25. All were reduced to two-thirds size in reproduction.

FIGS. 1-18. *Cassia auriculata*: (a) Type I. Fig. 1. Somatic metaphase, polar view ( $2n=14$ ). Fig. 2. Haploid set arranged seriatim. Fig. 3. Prophase showing the two satellited chromo-

But this pair behaved in step with the other chromosomes throughout the division stages.

Fig. 4 shows the two nucleoli organized at each pole at telophase, and Fig. 3 represents the two satellited chromosomes attached to the nucleolus at prophase. This suggests that the two nucleoli formed at telophase have been organized at the locus of the organizer in the satellited pair, since the satellites are attached to the nucleolus at prophase. Such a relationship was first shown by Heitz (1931 *a* and *b*) in several plants such as *Crepis*, *Vicia*, *Allium*, *Aloe*, and confirmed by several investigators following him, either directly from observations on the intimate connexions of the nucleoli with the satellited chromosomes, or indirectly from observations on numerical parallelism between the nucleoli formed at telophase and the SAT-chromosomes in the complement. The whole question is discussed elsewhere in this paper.

(*b*) Type II ( $2n = 16$ ). As stated before, one plant was found in the culture which exhibited a more vigorous vegetative growth. An examination of the root-tips showed  $2n = 16$  in this plant (Fig. 5), instead of the usual 14. Fig. 6 shows the haploid set arranged according to their lengths. A comparison with Fig. 2 (Type I) shows that two of the shortest chromosomes are reduplicated in the diploid complement. Thus, if the constitution of type I where  $2n = 14$  is

A	A	E	E
B	B	F	F
C	C	G	G
D	D		

the constitution of type II with the two reduplicated chromosomes will be

A	A	E	E		
B	B	F	F		
C	C	G	G	G	G
D	D				

This was verified by measurement of the actual lengths of the individual chromosomes, thereby proving conclusively that the shortest pair G G in type I was reduplicated in type II.

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 some attached to the nucleolus. Fig. 4. Telophase showing two nucleoli at each pole. (*b*) Type II. Fig. 5. Polar view of somatic metaphase ( $2n = 16$ ). Fig. 6. Haploid set arranged according to lengths. Fig. 7. Prophase showing the two satellited chromosomes attached to the nucleolus. Fig. 8. Telophase showing two nucleoli at each pole. *C. Tora*: Fig. 9. Polar view of somatic metaphase ( $2n = 28$ ) (many chromosomes paired). Fig. 10. Telophase showing two nucleoli. Fig. 11. Prophase showing two chromosomes attached to the nucleolus. Fig. 12. A cell with 56 chromosomes ( $8n$ ). Fig. 13. A cell with 42 chromosomes ( $6n$ ). Fig. 14. Prochromosomes, an early stage. Fig. 15. Prochromosomes, a slightly later stage. *C. javanica*: Fig. 16. Metaphase showing 28 chromosomes (note the two satellited chromosomes). Fig. 17. Prophase showing the two satellited chromosomes attached to the nucleolus. Fig. 18. Telophase showing two nucleoli at each pole.

Type II may have originated from type I by non-disjunction, producing a trisomic with  $2n = 15$ . This trisomic in the subsequent reduction division will produce gametes with eight and seven chromosomes. Union of two 8-chromosome gametes of the trisomic will result in a plant with 16 chromosomes, where two chromosomes are reduplicated. Or this type may have originated by parthenogenesis from an egg cell having eight chromosomes which arose as a result of non-disjunction. This type could also be formed directly by the union of two 8-chromosome gametes, both having arisen from type I, due to non-disjunction.

At telophase two nucleoli are formed (Fig. 8), and the two satellited chromosomes in the complement are attached to the nucleolus at prophase (Fig. 7).

In this case the organization of the nucleolus is interesting. At early telophase, in the preparations stained with the Feulgen-fast green technique, a number of minute diffused green particles are seen inside the nuclear membranes, which probably represent the matrix of the chromosomes contributing material towards the organization of the nucleolus. The aggregation of these 'diffused particles' at the locus of the nucleolar organizer in the SAT-chromosomes could not be made out since the chromosomes are small. They did not aggregate into bigger lumps, but at the next stage two finished nucleoli were seen. This suggests that the accumulation of the nucleolar matter at the locus of the organizer in the SAT-chromosomes is in this species a rapid process.

## 2. *C. Tora* L. ( $2n = 28$ ).

Fig. 9 shows a polar view of the somatic metaphase showing twenty-eight chromosomes. Satellites could not be made out because of the smallness of the chromosomes, but at telophase two nucleoli were formed (Fig. 10). This suggests that there are only two SAT-chromosomes in the complement and Fig. 11 shows two chromosomes attached to the nucleolus at prophase.

Datta (1933, 1934) and Senn (1938) reported  $n = 13$  in this species. The present count of the somatic chromosomes invariably showed  $2n = 28$ . This discrepancy cannot be explained unless it is assumed that of the thirteen bodies figured by Senn at metaphase I, one is a quadrivalent. On the assumption that *C. auriculata* ( $2n = 14$ ), with its two nucleoli at telophase and two satellited chromosomes attached to the nucleolus at prophase, is a diploid, *C. Tora* should be a tetraploid. Quadrivalent formation is the rule in autotetraploids unless the chromosomes are structurally changed, and even in allotetraploids occasional quadrivalents may be formed if there is some homology between the parental chromosomes.

Fig. 12 shows a tetraploid cell with fifty-six chromosomes; the conditions leading to this are dealt with elsewhere in this paper. If *C. Tora* with  $2n = 28$  is a tetraploid, this cell will be octoploid. Fig. 13 shows a cell with forty-two chromosomes (hexaploid). The satellited chromosomes could not be made

out. A  $6n$  cell in a tetraploid is extremely rare and it has been reported, as far as the writer is aware, only in one instance, *Calceolaria* (Srinath, 1939). In the present material, such a cell was found only once and its origin is not quite clear. In seeking to explain the origin of somatic doubling in five genera of the Chenopodiaceae, Lorz (1937) suggested a modification of de Litardière's idea of two successive cleavages of the chromosomes in prophase. While normally there is perfect co-ordination of the nuclear and the chromosomal divisions, Lorz thinks that under certain conditions this co-ordination may be upset. If some of the chromosomes in a mitotic complement undergo two cleavages in the prophase and arrive at the equator before the others have undergone one cleavage, aneuploid numbers may result. The present case can be explained on a similar basis if we assume that fourteen chromosomes (two genomes) undergo double cleavage and arrive at the metaphase plate while the other fourteen have undergone only one split. A cell division at this critical time would result in a cell with forty-two chromosomes.

*Prochromosomes* were observed in the resting cells of the root-tips. There was an approximate numerical relation between the number of prochromosomes present in a cell and the diploid complement of the plant. Fig. 14 shows an early stage where the prochromosomes are seen as rounded chromatin masses (positive to Feulgen). Fig. 15 shows a slightly later stage where these chromatin bodies are longer, and various gradations between this stage and the ultimate stage where the chromosomes are fully developed could be traced, suggesting that the prochromosomes really represent median portions of the chromosomes.

Prochromosomes have been observed in various plants. Rosenberg (1904) showed them for the first time in plants like *Capsella* and *Cucurbita* where there was an approximate numerical equality between the prochromosomes and the chromosome numbers in the cells. Different names have been given to these chromatic bodies by various authors, but the term prochromosomes first suggested by Overton (1905) is in wide use. Rosenberg and Overton held that prochromosomes were the centres around which the chromosomes were organized during prophase. Malte (1909), working with various members of the Euphorbiaceae, observed that the prochromosomes in the resting cells corresponded with the chromosome numbers, and concluded that prochromosomes were the chromosomes themselves. Stout (1912), in *Carex aquatilis*, also adopted the view of the structural identification of the prochromosomes and the chromosomes. Grégoire (1907, 1932), Heitz (1929), and others consider the prochromosomes as portions of the chromosomes, the spindle attachment regions, which persist through telophase. Smith (1934) in *Impatiens* finds that the number of prochromosomes in a cell may vary as the region becomes more achromatic. He finds no pairing or fusion of the prochromosomes in either somatic or post-meiotic nuclei, contrary to the observations of Rosenberg (1904), Overton (1909), and others; he also concludes that the prochromosome really represents a portion of the chromosome

on either side of, and adjacent to, the spindle attachment region. He does not agree with Grégoire (1932) and Doutrelinge (1933), who hold that chromonemata are not present in plants with prochromosomes. He thinks that in plants with prochromosomes the structure of chromosomes and the chromosome cycle in somatic and meiotic divisions is fundamentally the same as for plants without prochromosomes. Manton (1935) in *Biscutella laevigata* finds a numerical correspondence between prochromosomes and the chromosome numbers in the small nuclei which she calls 'vesicular nuclei'. In these nuclei a relatively enormous nuclear volume is attained by the accumulations of cell sap between the chromosomes, and in telophase extensive chromosome movements are involved, facilitating the formation of the prochromosomes. On the other hand, nuclei with relatively large chromosomes are 'solid', rich in chromatin and devoid of free cell sap until prophase, so that no chromosome movements take place in them. Raghavan (1938) in Capparidaceae thinks that the prochromosomes are directly derived from the telophasic chromosomes by the persistence of the chromatic material in the attachment constriction regions. He does not accept the theory of the pairing of the prochromosomes, since he finds the full diploid number in the resting stage and in telophase of the last premeiotic division. He finds no essential difference in the chromosome structure and chromosome cycle between plants with and without prochromosomes. Iyengar (1939) in *Cicer* also finds that the prochromosomes continue from telophase and persist in the resting nucleus as chromatic masses on either side of the spindle attachment region.

On the other hand, Lundegårdh (1912) holds that the prochromosomes bear no direct relation to the chromosomes. Kuhn (1929) working on *Capparis spinosa* also finds that the prochromosomes are not directly concerned in the formation of the prophasic chromosomes.

The present observations in *C. Tora* and other species of *Cassia* showed a numerical correspondence between the prochromosomes and the chromosome numbers. Pairing of the prochromosomes was not noticed in the resting stage. No differences were observed in the chromosome structure and chromosome cycle between species which show and those (*C. auriculata*) which do not show prochromosomes. There is no doubt that the prochromosomes really represent chromatic regions of the chromosomes and that the individuality of the chromosomes is never lost.

### 3. *C. javanica* L. ( $2n = 28$ ).

Fig. 16 shows a polar view of the somatic metaphase with twenty-eight chromosomes, of which two are satellited. Somatic pairing is very pronounced, the conditions leading to which are dealt with elsewhere (Jacob, in press). Fig. 18 shows two nucleoli at telophase and Fig. 17 shows the two satellited chromosomes attached to the nucleolus at prophase.

Thus *C. javanica* has only two nucleolar chromosomes, both of which are satellited. Similarly, *C. Tora*, *C. grandis*, and *C. floribunda* also have only two

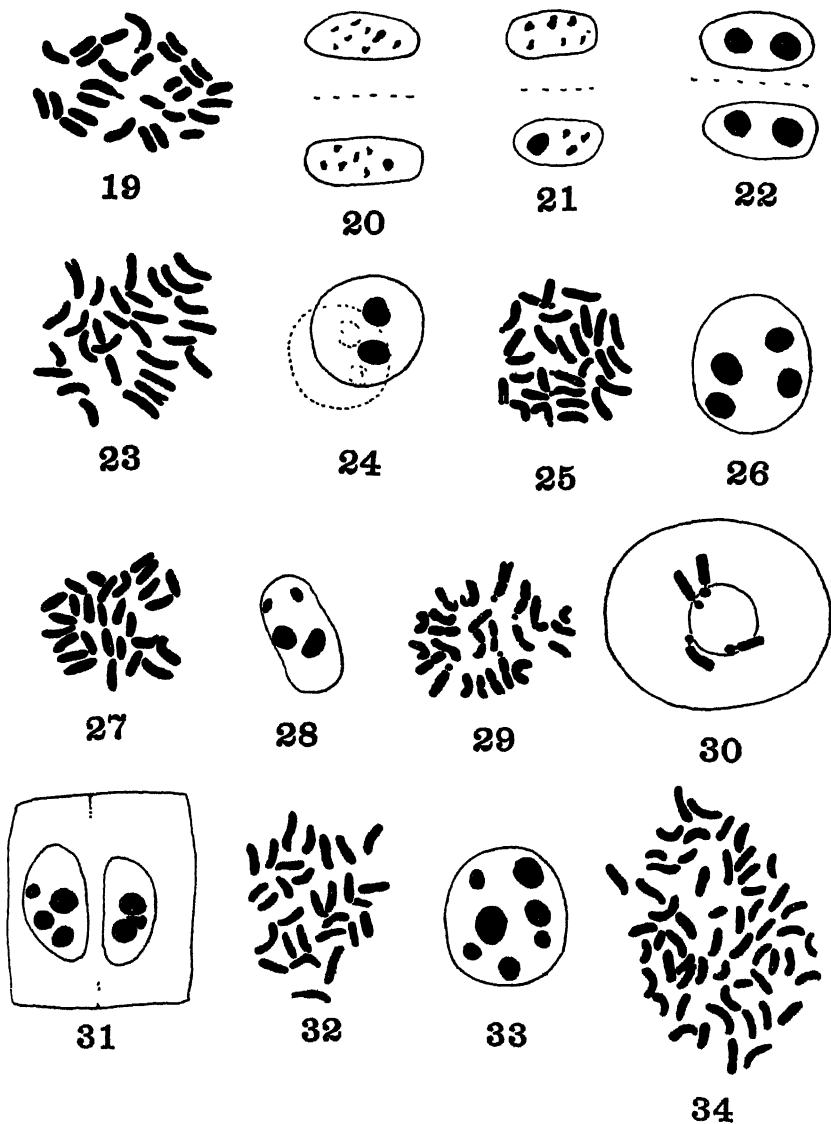
nucleolar chromosomes each, although their diploid complement consists of twenty-eight chromosomes. Starting on the assumption that in a normal diploid definite bodies located in a specific pair of chromosomes organize nucleoli at telophase, it is interesting to consider the corresponding changes in the chromosome complement. De Mol (1926) found two, three, and four nucleoli in the root-tips of  $2n$ ,  $3n$ , and  $4n$  plants respectively in *Hyacinthus*. Ramanujam (1937) in an autotriploid rice found three nucleoli, while the corresponding diploid had two. Bhatia (1938) observed four nucleoli in a tetraploid and six in a hexaploid wheat. These facts show that the number of nucleoli is an indication of the number of haploid complements present in the organism, unless the satellited pair alone has been duplicated. On the assumption that *C. auriculata* ( $2n = 14$ ), with its two satellited chromosomes attached to the nucleolus at prophase and its two nucleoli at telophase, is a diploid, *C. javanica*, *C. Tora*, *C. grandis*, and *C. floribunda*, where  $2n = 28$ , are tetraploids, and should show four nucleolar chromosomes organizing four nucleoli at telophase. But as stated above, all these show only two nucleoli. This shows that in the evolution of all these species two satellites or two nucleolar organizers have been lost. Similar cases have been reported in other plants. Navashin (1927, 1934) found in certain hybrids of *Crepis* that a satellite normally present in one of the contributing complements did not appear in the hybrid. This phenomenon, termed by him amphiplasty, may be interpreted as due either to the inability of the nucleolar body to function in a new environment or to competition between the nucleolar chromosomes in the organization of the nucleolus, resulting in the apparent inability of one of them to function. Similarly, Babcock *et al.* (1937) figured four species of *Lactuca* where  $2n = 16$  chromosomes; three of these are shown as having four SAT-chromosomes, while one has only two. It therefore appears likely that these species are secondary tetraploids of amphidiploid origin, and that *L. tenerima* has since lost one pair of satellites (Gates, 1938). Warmke and Johansen (1935) have found, in two related species of *Trillium* with the same chromosome number, that one of them has a satellite while the other lacks it. It thus appears that the mutational loss of a satellite is a frequent occurrence.

#### 4. *C. grandis* L. ( $2n = 28$ ).

Fig. 19 shows a polar view of the somatic metaphase with twenty-eight chromosomes, two of which are satellited. Many of the chromosomes exhibit somatic pairing.

The organization of the nucleoli is interesting. In the early telophase a number of small 'angular bodies' (up to eight) appear inside the nuclear membrane, which probably represent the matrix of the chromosomes contributing material towards the formation of the nucleoli (Fig. 20). The localization of these bodies at definite positions on the chromosomes was not seen, owing probably to the smallness of the chromosomes. At a later stage the number of these 'angular bodies' inside the nuclear membrane is considerably





FIGS. 19-34. *C. grandis*: Fig. 19. Polar view of somatic metaphase ( $2n = 28$ ) (many chromosomes show somatic pairing). Fig. 20. Early stage in the organization of the nucleolus (note the angular bodies). Fig. 21. A later stage, where some of the angular bodies have fused. Fig. 22. A still later stage showing the two finished nucleoli at each pole. *C. floribunda*: Fig. 23. Polar view of metaphase ( $2n = 28$ ). Fig. 24. Telophase showing two nucleoli at each pole. *C. siamea*: Fig. 25. Metaphase showing 28 chromosomes (4 chromosomes are satellited). Fig. 26. Telophase showing four nucleoli. *C. corymbosa*: Fig. 27. Polar view of somatic metaphase ( $2n = 28$ ). Fig. 28. Telophase showing four nucleoli, two larger and two smaller. *Caesalpinia pulcherrima*: Fig. 29. Polar view of somatic metaphase showing 24 chromosomes of which four are satellited. Fig. 30. Prophase showing the four satellited chromosomes attached to the nucleolus. Fig. 31. Telophase showing four nucleoli. *Poinciana regia*: Fig. 32. Metaphase showing 28 chromosomes. Fig. 33. Telophase showing seven nucleoli. Fig. 34. Metaphase showing 56 chromosomes.

reduced, while at the same time there is an increase in volume of some of them. This suggests that they are fusing together to form bigger masses. Fig. 21 shows a stage where three small 'angular bodies' and a much bigger 'globule' are seen at one pole, while at the other pole about six 'angular bodies' are still visible. They stain with the fast green. At a later stage two large finished nucleoli are seen (Fig. 22), probably due to the absorption of all these bodies and subsequent nucleolar growth.

5. *C. floribunda* Cav. ( $2n = 28$ ).

Fig. 23 shows the polar view of a somatic metaphase with twenty-eight chromosomes. They are longer than in the other species of *Cassia* described and some are paired. Satellites were not seen, but Fig. 24 shows the two nucleoli formed at telophase, which suggests that there are two nucleolar chromosomes in the complement.

6. *C. siamea* Lam. ( $2n = 28$ ).

Fig. 25 shows a polar view of the somatic metaphase with twenty-eight chromosomes. Four satellited chromosomes are seen and some of them are somatically paired. Fig. 26 shows four nucleoli at telophase, which corresponds with the four satellited chromosomes in the complement. This is in harmony with De Mol's hypothesis as well as the observations on *C. auriculata*. If *C. auriculata* ( $2n = 14$ ) is a diploid with two satellited chromosomes in the complement, and two nucleoli at telophase, then *C. siamea* should be a tetraploid with four nucleolar chromosomes producing four nucleoli at telophase. This is precisely the case in the present material.

7. *C. corymbosa* Lam. ( $2n = 28$ ).

Fig. 27 shows the polar view of a somatic metaphase with twenty-eight chromosomes. No satellites could be made out, but at telophase four nucleoli (Fig. 28) are formed, which suggests the presence of four nucleolar chromosomes in the complement.

Of the four nucleoli formed at telophase, two are smaller than the others. Similar variation in the size of the nucleoli has been noticed in other plants. Lesley (1938) in three races of *Solanum lycopersicum* noticed a difference in length of the nucleolar chromosomes (short A, long A, and very long A) due to the addition of satellite material. She observed that the nucleolus on an average is larger in races with two long A chromosomes than in races with two short, and still larger in the single plant with two very long A chromosomes. Thus there is a direct correlation between the size of the satellite and the size of the nucleolus formed. In *Sesbania bispinosa* (Jacob, in press) also, such a relationship was observed. On the other hand, McClintock (1934) in *Zea* correlated this variation in size of the nucleoli with the differences in the functional capacity of the nucleolar organizing element. Heitz (1931a) thought that this depended on the length of the satellite thread. Matsuura (1938)

refutes Heitz's contention on the ground that the size of the developing nucleolus is responsible for the length of the satellite stalks, since the larger the nucleolus the longer the thread will be stretched. Fischer (1934) is of opinion that the size of the nucleolus is largely dependent upon the physiological conditions of the cell (quoted from Matsuura, 1938). Until more light is thrown on the subject no definite conclusions can be drawn.

8. *Caesalpinia pulcherrima* Swartz ( $2n = 24$ ).

Fig. 29 shows the polar view of a somatic metaphase. Four satellited chromosomes could be made out in the complement. The chromosomes are small and are mostly medianly constricted. Fig. 31 shows the four nucleoli formed at telophase. As in *Cassia corymbosa*, they differ in size, although the satellites appear to be the same size. Fig. 30 represents the four satellited chromosomes attached to the nucleolus at prophase. Senn (1938) has figured the diploid complement in this species without showing any satellited chromosomes.

9. *Poinciana regia* (Boj.) Hook. ( $2n = 28$ ).

Fig. 32 shows the polar view of a somatic metaphase with twenty-eight chromosomes. The satellites could not be made out as the chromosomes were small. Somatic pairing is marked in many of the cells at this stage. The number and mode of attachment of the chromosomes to the nucleolus at prophase could not be made out, but at telophase seven bodies resembling nucleoli were found (Fig. 33). There is a marked variation in size between these nucleoli. If the number of nucleoli at telophase is an indication of the number of chromosome sets in a species, *P. regia* with its seven nucleoli at telophase should be a heptaploid. It also suggests that the basic number of this genus is four; this is in conformity with the observations of Wanscher (1934), who suggests that this is the basic number in Leguminosae. Iyengar (1939) in *Cicer* and the author (Jacob, in press) in *Sesbania* also arrived at the same conclusion. In the present material, the basic number can be confirmed only by a study of the attachments of the chromosomes to the nucleolus at prophase in mitosis and meiosis, and from the secondary associations of the bivalents at metaphase I and of the chromosomes at metaphase II. This was not possible as material was not available.

However, it should be pointed out that in rare cases the nucleoli may secondarily increase in number, especially so in those cells which possess an increasing vegetative function. Examples of this are known to occur in oocytes of *Patella coerulea* (Jørgensen), *Cyprinotus incongruens* (Geitler, 1934, quoted by Sato, 1936). Again Heitz (1931b) in *Vicia faba* and *Vicia monanthus* finds that certain lagging chromosomes produce their own nucleoli.

Occasionally in the roots a cell was seen with double the somatic number of chromosomes (Fig. 34). Somatic doubling in the cells of the roots is now a recognized phenomenon, being found in a number of plants like *Spinacia*

*oleracea* (Litardièrre, 1923), *Solanum lycopersicum* (Lesley, 1925), *Cicer* (Iyengar, 1939), &c. In some plants the cells with the increased number of chromosomes may form a definite sector in the root, as in *Crepis* (Navashin, 1926), and *Nicotiana* (Ruttle, 1928).

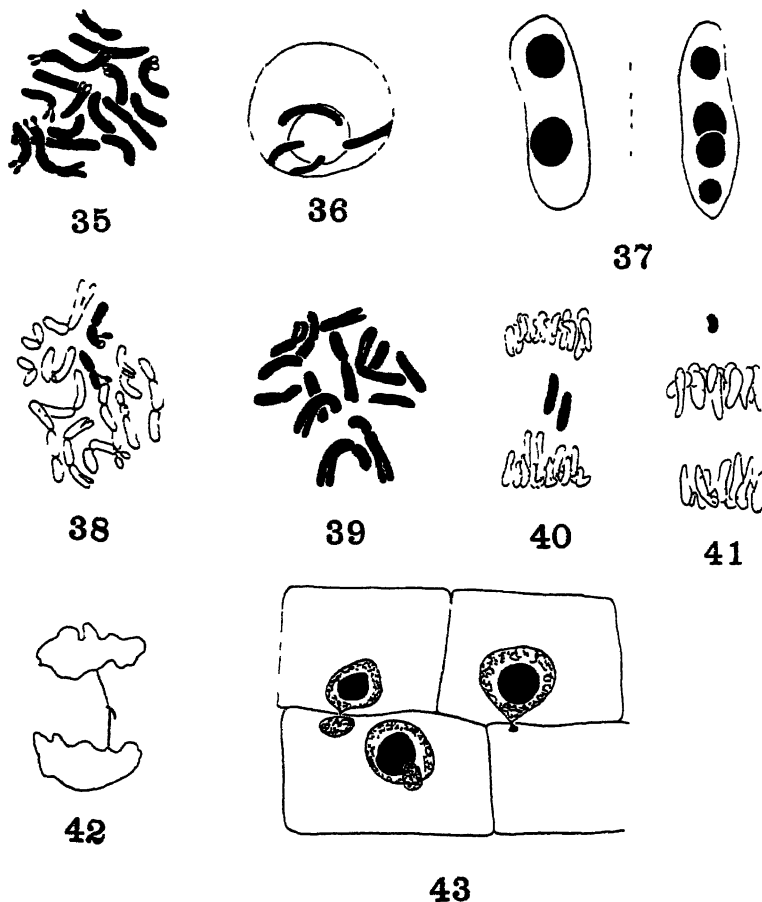
Somatic pairing is much more pronounced in the diploid cell (Fig. 32) than in the tetraploid cell (Fig. 34). Usually in syndiploid nuclei pairing is more pronounced as the chromosomes are in a favourable position to exercise their attraction on one another. Metz (1916) reported in certain tetraploid cells in *Diptera* that the chromosomes were associated together in groups of four instead of in pairs. In the genus *Sorghum* Huskins and Smith (1932) observed in a tetraploid cell, which arose as a result of an injury to the root-tip, a closely paired condition of the chromosomes. The failure to observe a more pronounced somatic pairing in this tetraploid cell of *Poinciana regia* may be due to the inability of the chromosomes to move freely in the cell owing to their relatively high number.

#### 10. *Clitoria ternata* L. ( $2n = 16$ ).

Fig. 35 shows a polar view of the somatic metaphase with sixteen chromosomes, four of which are satellited. Fig. 36 shows a prophase with the four satellited chromosomes attached to the nucleolus. The satellites in the present species are fairly large, suggesting a very short segment of the chromosome. Fig. 37 shows a telophase with four nucleoli, and these are evidently formed in connexion with the four satellited chromosomes. *C. ternata* should therefore be a tetraploid. This also suggests that the basic number in this genus is four. The diploid complement also contains two pairs of secondarily constricted chromosomes which do not organize nucleoli. One pair of chromosomes in the complement is very short and there is an extreme variation in size of the chromosomes.

Fig. 38 shows a metaphase plate with two chromosomes having lateral satellites. In one case the lateral satellite is very clear; this is evidently due to the translocation of the satellite from a terminal position, probably by inversion. Lateral satellites have been reported in other plants such as *Sesbania grandiflora* (Jacob, in press), *Allium* (Levan, 1932), *Tradescantia* (Darlington, 1929), *Crepis* (Swezy, 1935). Mather and Stone (1935) in *Crocus* and Camara (1939) in *Aloe* and *Vicia* also observed lateral satellites in materials treated with X-rays.

Fig. 39 shows a metaphase plate with fourteen chromosomes instead of the usual sixteen. Such variations have been reported in other plants, and the whole question is dealt with elsewhere (Jacob, in press). Fig. 40 shows two chromosomes lagging at anaphase. Such lagging chromosomes may bring about sudden alterations in the chromosome number in mitosis, and it is probable that the cell with fourteen chromosomes described above may have originated in this way. Somatic lagging has been observed in other plants,



FIGS. 35-43. *Clitoria ternata*: Fig. 35. Polar view of somatic metaphase ( $2n = 16$ ) with four SAT-chromosomes, two longer and two shorter. Fig. 36. Prophase showing four chromosomes attached to the nucleolus, two longer and two shorter. Fig. 37. Telophase showing four nucleoli. Fig. 38. Metaphase showing two chromosomes having lateral satellites. Fig. 39. Metaphase with fourteen chromosomes. Fig. 40. Anaphase showing two lagging chromosomes. Fig. 41. Anaphase showing a fragment. Fig. 42. Somatic bridge with fragment. Fig. 43. Cytomixis in root-tip with resting nuclei.

such as *Sesbania Sesban*, *S. speciosa* (Jacob, in press), wheat (Plotnikova, 1932), and Cicer (Iyengar, 1939).

Occasional fragments were noticed (Fig. 41), the conditions leading to which are dealt with elsewhere (Jacob, in press). Fig. 42 shows a somatic bridge and a fragment at somatic anaphase; the fragment is lying across the bridge. It has been concluded elsewhere that this bridge may have originated by reciprocal translocations between two chromosomes, giving rise to a dicentric chromosome and one or two acentric fragments according to whether the fragments fuse or remain free.

Fig. 43 shows in root-tips the extrusion of chromatin from one cell to another. A similar condition was shown in *S. Sesban* (Jacob, in press), but it has not been reported in roots before. The transference of chromatin between two contiguous cells through gaps in the cell-wall by means of protoplasmic connexions was described by Gates (1911) in the pollen mother-cells of *Oenothera gigas*. This phenomenon, termed cytomixis by Gates, has been subsequently reported in a number of other plants and the whole question is discussed elsewhere (Jacob, in press).

#### IV. DISCUSSION

##### (a) *Nucleolus*.

The problem of the origin, behaviour, and composition of the nucleolus as well as its relation to the chromosomes has been the subject of numerous investigations from time to time, and a vast amount of literature has accumulated on the subject. The extensive literature concerning the nucleolus has been summarized by Ludford (1922), De Mol (1927), Sharp (1934). Gates (1937) has reviewed the relevant literature concerning the relationship between the nucleolus and the chromosomes.

Early investigators frequently noted in the plant cells some connexion between the nucleolus and the network of the nucleus. Thus Farmer (1895) from his observations on the pollen mother-cells in Hepaticae concluded that the nucleolus was associated with the chromosomes by means of a delicate thread. Wager (1904), in the root-tips of *Phaseolus*, regarded the nucleolus as suspended in the nuclear network by numerous strands. But the credit of establishing a definite morphological relationship is due to Latter (1926) who, in the pollen development of *Lathyrus odoratus*, demonstrated the presence of a peripheral area in the nucleolus which she termed the 'nucleolar body'. Such a fixed relationship between the nucleolus and the chromosomes was subsequently shown in the pollen mother-cells of other plants such as *Oenothera* (Sheffield, 1927; Gates and Sheffield, 1929), and *Lathraea* (Gates and Latter, 1927).

Another line of observations which led to a better understanding of the subject began with the discovery of satellites by Navashin (1912) in *Galtonia*, where he observed a pair of satellites attached at first to the nucleolus. Sorokine (1924, 1929) reported a pair of satellite chromosomes attached to the nucleolus in *Ranunculus acris* and *R. chius*. Senjaninova (1926) in *Ranunculus acer* also found a pair of satellited chromosomes attached to the nucleolus in the resting stage. Heitz (1931b) found that the number of nucleoli formed at telophase depended upon the number of SAT-chromosomes in the complement. The papers establishing a similar relationship are too many to be enumerated here; for a fuller review see Gates (1937 and 1938).

As regards the origin and organization of the nucleolus various theories have from time to time been put forward. Van Camp (1924) thought that the

nucleoli originated from the chromosomes at telophase in the form of small globules which later fused into larger forms, and still later during the development of the chromosomes, through a direct contact established between the 'continuous spireme' and a nucleolus, the nucleolar substance flowed back again over the chromosomes to be again dissociated during telophase. Zirkle (1928 and 1931) supported the idea of direct transport of the nucleolar substance into the 'spireme', and demonstrated the presence of this substance in a sheath around the anaphasic chromosomes. Heitz (1931*a* and *b*) is of opinion that the nucleoli originate from the SAT-chromosomes only, being organized around the thread of the satellite or at the secondary constriction. Resende (1937), from a study of 151 species of *Aloe*, also confirmed Heitz's findings. McClintock (1934) in *Zea* found a definite body on the chromosome at the base of the satellite stalk, which she termed the 'nucleolar organizing body', claiming that it was responsible for the organization of the nucleolus. Derman (1933) is of opinion that the nucleoli originate on the surface of the chromosomes in the form of small globules and not at a specific region—threads of the satellite or secondary constrictions. Matsuura (1938) in *Trillium kamschaticum*, in whose complement neither satellited nor secondary constricted chromosomes were seen, observed the development of the nucleoli at both distal ends of 'chromosome A' and the distal end of the short arm of 'chromosome E', but in *Paris hexaphylla* he observed that the nucleoli arose at the satellite stalk of 'chromosome D'. He came to the conclusion, from a study of the abnormal meiotic divisions in *Trillium*, that every chromosome of a complement is provided with functional activity for nucleolar development, which expresses itself in special conditions, as when single chromosomes are isolated and form a micronucleus. Gates and Pathak (1938) in *Crocus* observed that each of the nucleoli arises as two bodies close together, one on each arm of the twin threads of a telophasic chromosome. As these nucleolar rudiments grow they shortly fuse into one globular body which then grows to the full-sized nucleolus of the resting nucleus.

From a study of the organization of the nucleolus in the various species of *Cassia*, *Sesbania*, and other genera, the present writer is of opinion that the nucleolar material is derived from the matrix of all the chromosomes. At telophase these may be seen in the form of small globules as in *Sesbania grandiflora*, or as diffused particles dispersed in the nuclear sap as in *Cassia auriculata*, or in the form of small angular bodies as in *C. grandis*, which later on contribute to form the finished nucleoli. The process of nucleolar development thus appears to be a surface phenomenon, which can be explained by assuming that a chemical reaction takes place between the substance on the surface region of the chromosome (matrix) and the nuclear sap. The attachment of the satellited as well as the secondarily constricted chromosomes to the nucleolus at prophase in the various species shows that the independent nucleoli are organized at these loci at telophase, and later fuse to form the compound nucleus seen in the resting stage. From this it can be inferred

that these loci exercise some special attraction in collecting the nucleolar material, which also suggests the presence of a nucleolar organizing body, as seen by McClintock at the base of the satellite stalk in chromosome VI of *Zea mays*. Such a body was seen only in the secondarily constricted chromosome in *Sesbania speciosa*, but the failure to observe this is no proof of its absence, especially as these nuclei are very small.

Although the nucleolus is an essential component of the cells, since it is very probably present in all types of organisms, its function has been and is still a matter of speculation. Montgomery (1898) assumed that the nucleolar substance is connected with the nutritive process of the cell and as an assimilation organ takes food from the cytoplasm. It is believed by many that the nucleolus is nothing but reserve material for the chromosomes, which may build themselves from this supply. De Mol (1926), Zirkle (1928), and Fikry (1930) suggested that this body may break up into small particles and act as the carriers through the cytoplasm of hereditary materials from the genes. Derman (1933) assumed that the nucleolus is concerned in the general metabolic process of the organism as a whole. Nandi (1937) thinks that since the nucleolus is a compound product consisting of substances from all the chromosomes, and hence perhaps from all the genes, it may have a certain role in inheritance. From our present knowledge of the nucleolus there is no doubt that it is something more than an assimilation organ or a storehouse of reserve food. But there is no reason for attributing genic qualities to the nucleolus because the nucleolar matter is derived from the matrix of the chromosomes, especially as the matrix is held to be a non-genic portion of the chromosome. The exact function of the nucleolus in the economy of the cell remains imperfectly understood.

Francini (1938), from a study of the root-tips in *Paphiopedilum spicerianum*, fixed by twelve different methods, came to the conclusion that the nucleolus is of dual composition; (a) a protein portion not stained by Heidenhain's hematoxylin and remaining constant in bulk through prophase, and (b) a readily stained portion (diminishing through these stages) which is a sulphuric ester of a polysaccharide and resembles mucilage or pectin in its behaviour towards reagents and in its intense staining with ruthenium red. Fixatives containing acetic acid destroy it, and so do successive washings. During prophase the mucilaginous substance passes into the karyolymph and appears again with the reconstruction of new nucleoli, at first as small isolated granules which later become aggregated in the nucleoli. The present observations do not lend any support to the view that the stainable portion of the nucleolus is destroyed by fixatives containing acetic acid; there was no appreciable change in the volume of the nucleolus in materials fixed in (a) acetic alcohol, (b) Navashin's fluid, (c) Levitsky fluid. Yet (a) and (b) contain acetic acid, and (c) is devoid of it. Also Feulgen tests indicate that protein is generally absent from the nucleolus. Microchemical tests carried out by Yamaha and Sinoto (1925), Zirkle (1928, 1931), &c., indicate that the nucleolar



substance is not composed chemically of 'nucleic acid', 'chromatin' or 'karyotin'. Until more light is thrown on the subject, no definite conclusions can be drawn. Mensinkai (1939,) found evidence of the presence of lipoids in the nucleolus.

(b) *Speciation.*

From a general survey of the four genera in the present investigation, the changes in the chromosome complements differentiating species may be inferred as changes in (1) chromosome length, (2) chromosome number, (3) chromosome morphology, (4) structural changes in the chromosome such as inversion, fragmentation, fusion, &c.

In a number of instances, differences between species and their varieties have been attributed to differences in the size of the chromosome complements. Two clones of *Fritillaria ruthenica* (Darlington, 1936) were found to differ in the size of the chromosomes, the smaller type having smaller chromosomes than the other. In many cases, such a change in the size of the chromosomes of the entire complement is genotypic, as in *Melandrium* (Breslavetz, 1929), *Lolium perenne* (Thomas, 1936), *Oryza* (Ramanujam, 1938). Lesley and Frost (1927) in *Matthiola* have shown that the length of the chromosomes at the first meiotic divisions is controlled by the action of a gene. This is significant in connexion with the fact that the different sizes of the chromosomes in related species may be genotypically controlled.

Changes in number of the chromosomes in related species may be in multiples of a basic set. This phenomenon of polyploidy has been of considerable interest ever since Winge (1917) pointed out the high frequency of multiples and the low frequency of primes among the gametic chromosome numbers in flowering plants. A reference to Gaiser's (1930) and Tischler's (1935-6) lists of chromosome numbers reveals the very high percentage of polyploid series in the various families and genera. Gates (1924), Müntzing (1933, 1936) and others have considered this phenomenon.

At least in polyploids which have arisen recently an increase in cell size is generally correlated with an increase in chromosome number. Gates (1909) in *Oenothera gigas* found a corresponding increase in the nuclear volume. Müntzing (1927-8) found in *Galeopsis* that 16-chromosome species had larger nuclei, pollen mother-cells and pollen grains than the 8-chromosome species. Hagerup (1932) reported the pollen mother-cells of *Euphorbia granulata* ( $n = 20$ ) to be larger than those of *E. scordifolia* ( $n = 10$ ). Levan (1932) measured the pollen of certain diploid and tetraploid species of *Allium* and found the average length of the pollen of the former to be  $29\mu$  and the latter  $35\mu$ . Gershoy (1934) found that within the related species groups of *Viola*, there was a definite tendency for increased nuclear and pollen grain size to accompany an increase in chromosome number. Babcock *et al.* (1938) in *Crepis* observed that the size of the stomata increased with increasing polyploidy. They also found that the morphology of the pollen grains was

generally useful in determining the number of chromosome complements in a species; diploids having three pores and tetraploids four.

Autopolyploidy was distinguished from allopolyploidy by Kihara and Ono (1927). There is no doubt that in nature both allo- and autopolyploids have played an important part in the evolution of the species. The first autotetraploid described arose as a mutant in a culture of *Oenothera*, perhaps through a longitudinal split in the chromosomes in the fertilized egg (Gates, 1909). Müntzing (1936) lists about fifty-eight instances where, in nature, polyploids arose from their diploid ancestors. In autopolyploids the presence of more than two homologous chromosomes leads to multivalent formations unless some sort of genetic control or structural differentiation in the chromosomes ensures bivalent formation. Claussen (1926), Wettstein (1928, 1932), and Nandi (1936) consider that autopolyploidy is of no evolutionary significance, due to the difficulty of explaining the mechanism by which quadrivalent formation could be suppressed. Structural changes, like inversion, translocation, deletion, and segmental interchange, can effectively prevent quadrivalent formation, although its wide occurrence in nature is not clearly understood.

There is no doubt that allopolyploidy has played a greater part than autopolyploidy in the evolution of species. This type of duplication arises by the hybridization of two different species, with subsequent doubling of the chromosomes. Allopolyploids have been obtained experimentally in a number of cases. *Primula Kewensis* ( $n = 18$ ) came from a cross between *P. floribunda* ( $n = 9$ )  $\times$  *P. verticillata* in which a branch arose with twice the number of chromosomes (Newton and Pellew, 1929). Other examples are *Nicotiana Tabacum-sylvestris* (Rybin, 1929); Raphano-Brassica (Karpechenko, 1927), &c.

Allopolyploids have also been known to occur under natural conditions, such as *Galeopsis Tetrahit* (Müntzing, 1930, 1932); *Spartina Townsendii* (Huskins, 1931). It is not always possible to determine the allopolyploid origin in old allopolyploids, as structural changes and genic rearrangements make it difficult to trace back the individual chromosomes, but *Oryza minuta* (Nandi, 1936), *Sesbania bispinosa* (Jacob, in press), and many others are probably of this nature.

In addition to the balanced duplication of whole sets, unequal duplications have been inferred in some genera. Thus some of the so-called diploids have been found to be secondarily derived from a smaller basic set of chromosomes. Moffett (1931) in Pomoideae assumed that the diploid set of seventeen and its multiples had been derived from a basic set of seven. In *Oryza*, the diploid set of twelve has been inferred to have been derived from a basic set of five (Sakai, 1935, and Nandi, 1936). Parthasarathy (1939) thinks that the haploid set of twelve in *Ehrharta* is derived from a basic set of five.

Certain genera, *Cassia*, *Vicia*, *Brassica*, show aneuploidy, which unlike polyploidy is not a common phenomenon in the angiosperms. Although the species of *Cassia* under investigation showed apparent diploids with  $2n = 14$

and apparent tetraploids with  $2n = 28$  chromosomes, a number of other species are reported with aneuploid numbers. Thus *Cassia fistula* (Tischler, 1921-2) has  $2n = 24$ ; *C. occidentalis* (Muto, 1929) has  $2n = 26$ ; *C. sophora* (Kawakami, 1930) has  $2n = 24$ ; *C. dimidiata* (Sugiura, 1931) has  $2n = 16$  chromosomes. Manton (1932) found in the Cruciferae that aneuploidy was a common relation between genera, but not between species within the genus. An aneuploid relationship between incipient genera may produce an effective barrier to interbreeding, which will permit subsequent specific differentiation through genic changes and polyploidy as well (Senn, 1938). In Leguminosae aneuploidy plays an important part in intergeneric relationship. Although aneuploidy in this family is a common method of generic origin it is by no means the only one. A few genera are entirely polyploids, and a considerable number of genera, especially within closely related groups, have the same chromosome number, indicating that generic differences must be due to differences in chromosome size, chromosome morphology, or genotypic constitution.

## V. SUMMARY

The chromosome numbers as well as the relationship between the SAT-chromosomes and the nucleoli have been investigated in ten members of the Leguminosae, i.e. seven species of *Cassia*, and one species each of *Cæsalpinia*, *Poinciana*, and *Clitoria*. The results are embodied in Table I, p. 205.

In the culture of *Cassia auriculata* ( $2n = 14$ ) one larger plant was found in which the shortest pair GG was reduplicated. This type may have arisen by the union of two 8-chromosome gametes formed by non-disjunction.

Loss of two satellites or two nucleolar organizers has been inferred in *Cassia Tora*, *C. javanica*, *C. grandis*, and *C. floribunda*; these are tetraploids with  $2n = 28$ , but having two nucleolar chromosomes only.

In *C. Tora* a single cell was found with forty-two chromosomes. This cell may have arisen by double cleavage of fourteen chromosomes (two genomes).

Prochromosomes were observed in all species of *Cassia* except *C. auriculata*. There was a numerical equality between them and the diploid chromosome complements.

*Poinciana regia* ( $2n = 28$ ) has seven nucleoli at telophase and *Clitoria ternata* ( $2n = 16$ ) has four nucleoli at telophase. These and other results support the conclusion that the basic number in the Leguminosae is four.

Lateral satellites, somatic lagging, somatic bridge and fragment, and cytotoxicity in root-tips were also observed in *Clitoria ternata*.

From a study of the organization of the nucleolus in the various species and genera, using the Feulgen-fast green stain, it was concluded that the nucleolar material is derived from the matrix of all the chromosomes which becomes organized at the locus of the organizer in the SAT-chromosomes under the influence of the nucleolar organizing body.

From a general survey of the species and genera under investigation, the

changes in the chromosome complements differentiating species were inferred as changes in (1) chromosome length, (2) chromosome number, (3) chromosome morphology, (4) structural changes in the chromosomes. The problem of speciation is discussed.

In bringing the present series of papers to a close, I wish to express my most sincere gratitude to Professor R. R. Gates for his valuable help, guidance, and criticism during the progress of these investigations.

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# Studies in the Cytology of *Crocus*<sup>1</sup>

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With sixty-two Figures and three Diagrams in the Text

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## I. INTRODUCTION

THE genus *Crocus*, belonging to the family Iridaceae, consists of about eighty species as given in the *Index Kewensis*. The plants are usually propagated vegetatively by means of their corms, commonly called bulbs, and are used mostly for ornamental purposes. Due to variation in chromosome numbers (including plants even with odd chromosome numbers, which have survived probably by virtue of their possessing a successful method of vegetative propagation) as reported to date, the genus has furnished excellent materials for cytological studies.

Cytological studies in the genus were taken up in 1926 when Himmerbaur determined the somatic chromosome numbers of one and Heitz of six species. Later on Morinaga and Fukushima (1931) reported the somatic counts of two

<sup>1</sup> Part I of thesis accepted for the Ph.D. Degree of the University of London.



species, in one case confirming the result of Himmerbaur. The credit of reporting the somatic chromosome numbers of forty-three species and varieties goes to Mather (1932), who found haploid numbers ranging from 3 to 15. Even with such a great variation in chromosome numbers, he found very little evidence of polyploidy. He attributes the chromosome variation to (i) hybridization and polyploidy, (ii) fragmentation and possibly fusion, and (iii) genotype changes controlling the bulk and width of the chromosomes. From his figures the morphology of the chromosomes appears to be fairly clear. In a good many cases he has sketched satellites also, although he made no special study of these structures. Brittingham (1934) reported the chromosome numbers of six species, in each case confirming the counts of Mather.

Karasawa (1932, 1933, 1935, 1937, and 1939) has determined the somatic chromosome numbers and studied the meiotic stages of a good many species and varieties of *Crocus*. In fact, he is the only investigator who has studied the meiosis on a large scale until now. In the majority of cases he confirmed the counts of the previous workers and found haploid numbers ranging from 3 to 13. He traced two polyploid series, with eight and ten as basic numbers. He attributed sterility in the species to irregular meiosis in the microsporangous tissue, e.g. lagging and unequal distribution of the chromosomes, &c. Tetrads and seed-formation were found to be usually normal in many cases.

From the above-mentioned sketch of the previous work done in the genus, it is evident that the study of the relation of the SAT-chromosomes to the nucleoli has been completely neglected. The importance of such study was first stressed by de Mol (1926) in *Hyacinthus* where he found that the number of nucleoli in the root-tip cells was a reliable guide to the polyploidy of plants. A definite correlation between the number of SAT-chromosomes and nucleoli in a complement was, however, first established by Heitz (1931a, b). A number of investigators have found results confirming both the above-mentioned hypotheses, though exceptions are also present as is usually expected against any hypothesis. Recently Gates (1937, 1938) has reviewed the most important literature on this subject. In the later paper he has suggested

‘three complementary lines of observation to bear on the phylogeny of the nucleus in case of primary or secondary polyploidy; (1) the maximum number of nucleoli in telophase nuclei, as a preliminary indication of polyploidy, (2) the number of satellited chromosomes in somatic and meiotic mitoses, and any difference between their satellites as well as the number of bivalents attached to the nucleolus in zygotene to diakinesis; (3) the maximum secondary pairing of chromosomes in the first and second metaphase. When these results confirm each other, the evidence of former changes, even in the basic number of chromosomes, is on a firm basis.’

Gates and Pathak (1938) have already shown in *C. sativus* the bearing of the relation of SAT-chromosomes and nucleoli on the structure of chromosomes.

The object of the present paper is thus to study some *Crocus* species from

the above-mentioned points of view and thereby to find out the existence of any more polyploid series in the genus and also to throw some light on the original basic number.

## 2. MATERIALS AND CYTOLOGICAL TECHNIQUE

The bulbs of the following twelve species and two varieties of *Crocus* were obtained from Messrs. Barr & Sons, Ltd., London: (1) *Crocus Olivieri* Gay, (2) *C. zonatus* Gay, (3) *C. aerius* Herb. var. *Gray Lady*, (4) *C. ochroleucus* Boiss. and Gaill., (5) *C. susianus* Ker-Gawl., (6) *C. pulchellus* Herb., (7) *C. speciosus* Bieb., (8) *C. speciosus* var. *albus* Hort., (9) *C. Tomasinianus* Herb., (10) *C. Korolkowii* Maw. and Regel, (11) *C. Salzmannii* Gay, (12) *C. sativus* Linn., (13) *C. sativus* var. *Elwesii* Maw., (14) *C. Tournefortii* Gay.

The plants were grown in a greenhouse in pots with a mixture of half sand and half soil. Flower-buds at the right stage of division could be collected only from *C. ochroleucus*, and *C. susianus*. The following fixatives were used: (1) Benda's Fluid (low acetic), (2) 2BE, (3) Levitsky—1 per cent. chromic acid and 10 per cent. Formalin in the ratio 2 : 3, (4) Navashin's fluid.

Root-tips were fixed in all the above-mentioned fixatives, but for flower-buds only Navashin's fluid was used. The time for fixation was usually between 11 a.m. and 2 p.m. on a bright day which followed a similar condition on the previous day, to ensure the presence of normal activities in the plant cells. The materials were kept in the fixatives for about twenty-four hours, washed usually with frequent changes of tepid water, dehydrated by the usual chloroform method and embedded in paraffin. Sections of the root-tips were cut from 12 to 18  $\mu$  and those of the flower-buds 20  $\mu$  thick. For studying chromosome morphology sections were stained by the usual Newton's iodine gentian-violet method, but for studying the number of nucleoli in telophase and the relation of satellites to nucleoli sections from root-tips fixed in Navashin's fluid were differentially stained with Feulgen-light green after Semmens and Bhaduri (1939). Preparations by the root-tip smear technique after Bhaduri (1938) were also made with great success and proved after differential staining, to be superior to those of the sections particularly showing the nucleoli in telophase where they take a deep and bright stain.

## 3. GEOGRAPHICAL DISTRIBUTION OF THE SPECIES

From Table I (p. 231) it becomes evident that all the species studied are found in the regions surrounding the Mediterranean Sea and the majority of them in the countries towards the east. It appears that the typical Mediterranean climate favoured the origin, distribution, and even the preservation of the species. Most of them are now cultivated in different parts of the world.

## 4. OBSERVATIONS

## A. Somatic

*Chromosome numbers* (Table I).

The counts of the chromosomes made here agree with those of the previous workers practically in all cases except in *C. Tomasinianus* and *C. susianus*. The former has been reported to possess sixteen chromosomes by Mather (1932) and Brittingham (1934) as against that of Heitz, who found seventeen, eighteen, and nineteen chromosomes and even suggested that the plants with eighteen chromosomes may be triploid. Such variations of chromosome numbers have been observed in somatic cells by many investigators and may also be due to difference in the materials examined. Anyhow the number as found here tallies with those of the first two investigators mentioned. *C. susianus* has already been reported by Karasawa (1935) to be an autotriploid having fifteen chromosomes, presumably with five as basic number. But the present count shows that the species has twelve chromosomes. How this number has been derived can be explained in the following manner. The presence of a pair of SAT-chromosomes and corresponding number of nucleoli in telophase and prophasic attachment of satellites to nucleolus (Figs. 12, 13, 14, respectively) indicates that it is a diploid on the hypothesis that each SAT-chromosome and nucleolus represents a haploid complement. According to the triploidy observed by Karasawa, ten will be the diploid number. It appears that a reduplication of a pair of chromosomes, not those bearing the satellites, has taken place giving rise to the number twelve, and then it may be called a secondarily-balanced diploid. This finds enough support from the study of meiosis where (i) at diakinesis and metaphase I (Figs. 52, 54) along with four bivalents a ring of four chromosomes, which is probably formed by the four homologous chromosomes after reduplication, was observed; and (ii) one bivalent was found always attached to the single fused nucleolus at diakinesis and earlier stages (Fig. 50), proving not only the presence of a pair of SAT-chromosomes in somatic cells but also that SAT-chromosomes have not been reduplicated.

*Morphology of the chromosomes.*

The following signs have been adopted to elucidate the size of the chromosomes and the position of the primary, or spindle-fibre attachment constrictions: *L* = large, *M* = medium, *S* = small chromosomes; *m* = median, *sm* ≠ submedian, *st* = subterminal constrictions.

1. *C. Olivieri* ( $2n = 6$ , Fig. 1) 2 *Lst*+2 *Mst*+2 *Ssm*. Two secondary constrictions (nucleolar) are present on the short arm of the small pair of chromosomes. Karasawa (1937) described them as a pair of large satellites.

2. *C. zonatus* ( $2n = 8$ , Fig. 4) 2 *Lst*+2 *Mst*+4 *Sst*. Two satellites of unequal sizes are present on the short arm of one pair of small chromosomes.

Table I

Name of species.	Geographical distribution of species.	Chromosome numbers.	No. of satellites.	No. of secondary constrictions.	Maximum No. of nucleoli at telophase.	No. of attachments of satellites to nucleoli at prophase.	Reference to figures.
1. <i>C. Olivieri</i>	Greece to Asia Minor	6	—	2	2	2	1-3
2. <i>C. zonatus</i>	Asia Minor, Cilicia	8	2	—	2	2	4-6
3. <i>C. aeri</i> var. <i>Gray Lady</i>	Persia, Armenia, Kurdistan	8	4	—	4	—	7 & 8
4. <i>C. ochroleucus</i>	Syria	10	2	—	2	2	9-11
5. <i>C. susianus</i>	Asia Minor, Crimea	12	2	—	2	2	12-14
6. <i>C. pulchellus</i>	Greece to Asia Minor	12	4	—	4	4	15-17
7. <i>C. speciosus</i> var. <i>albus</i>	Shemacha in the Caucasus	12	4	—	4	4	18-20
8. <i>C. speciosus</i>	Tauria—S.E. Europe and Asia	18	6	—	6	6	21-23
9. <i>C. Tomasinianus</i>	Dalmatia, Servia	16	2	—	2	2	24-26
10. <i>C. Korolkowii</i>	Turkey, Asia Minor	20	4	—	4	4	27-29
11. <i>C. Salzmanii</i>	Morocco	24	2	2	4	4	30-32
12. <i>C. sativus</i>	Greece to Asia Minor	24	3	—	3	3	33, 34, 36, 37
13. <i>C. sativus</i> var. <i>Elwesii</i>	Near Smyrna	15	2	—	2	2	41-43
14. <i>C. Tournefortii</i>	East Europe	30	2	—	2	2	47-49

3. *C. aeri* var. *Gray Lady* ( $2n = 8$ , Fig. 7)  $2 Lsm + 2 Mst + 4 Sst$ . Four satellites are present, the largest one on the long arm of a long chromosome, two of unequal sizes on the short arm of the medium chromosomes, and one, which is equal to the smaller on the medium chromosomes, on the short arm of one small chromosome.

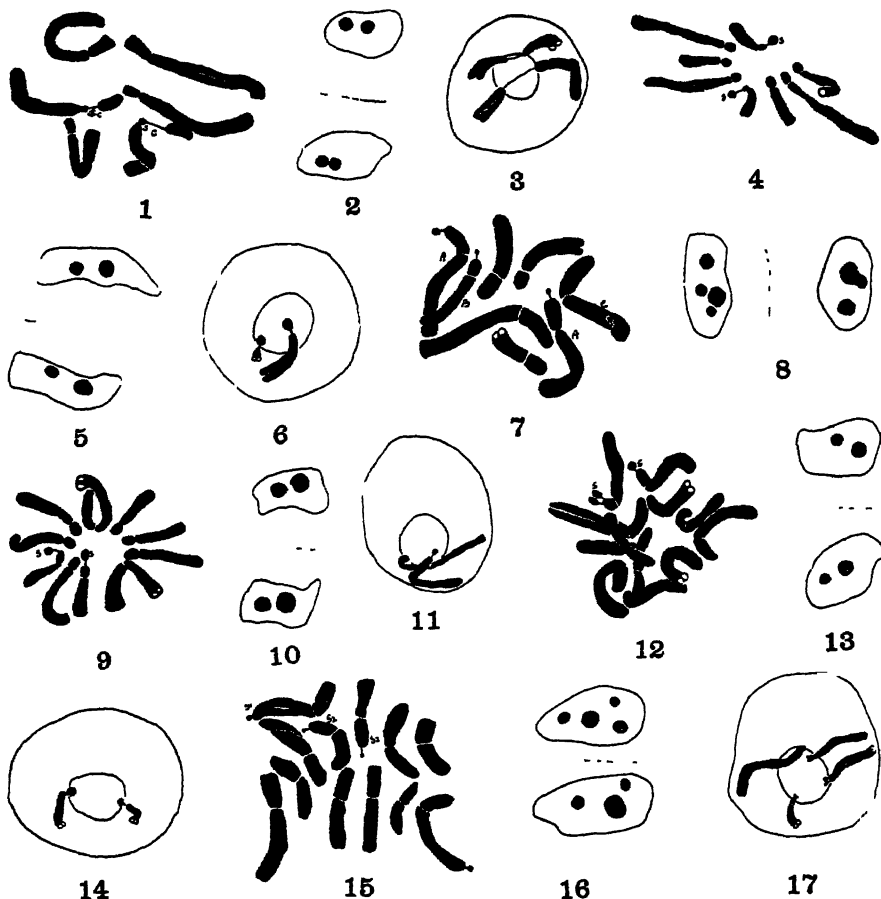
4. *C. ochroleucus* ( $2n = 10$ , Fig. 9)  $2 Lsm + 4 Mst + 4 Sst$ . Two satellites of unequal sizes are present on the short arm of a pair of small chromosomes.

5. *C. susianus* ( $2n = 12$ , Fig. 12)  $2 Lst + 4 Mst + 4 Ssm + 2 Sst$ . Two satellites of unequal sizes are present on the short arm of a pair of medium chromosomes.

6. *C. pulchellus* ( $2n = 12$ , Fig. 15)  $4 Lst + 4 Msm + 4 Ssm$ . Four satellites are present, two larger ones on the long arm of a pair of long chromosomes and the other two on the short arm of a pair of small chromosomes.

7. *C. speciosus* var. *albus* ( $2n = 12 = 4b$ , Fig. 18)  $4 Lsm + 2 Msm + 4 Mst + 2 Sst$ . Four satellites are present, two larger ones on the short arm of a pair of long chromosomes and the other two on a pair of medium chromosomes, but one on the short arm and the other on the long arm.

8. *C. speciosus* ( $2n = 18 = 6b$ , Fig. 21)  $6 Lsm + 8 Mst + 2 Ssm + 2 Sst$ . Six satellites are present, one pair on the long arm of a pair of long chromosomes, the second pair on the long arm of a pair of medium chromosomes, and the third pair on the short arm of a pair of medium chromosomes.



All drawings were made at bench level with an Abbe camera lucida and an achromatic objective N.A. 1.3. For all somatic metaphase figures a Zeiss ocular K30 $\times$  was used, giving a magnification of 4,050; for the rest of the figures except 60 and 61 a Beck ocular K25 $\times$ , giving a magnification of 3,050; for figures 60 and 61 a Zeiss ocular K15 $\times$ , giving a magnification of 2,070. All the drawings are reduced to half in reproduction.

FIGS. 1-17. *Somatic figures*. Fig. 1. *C. Olivieri*,  $2n = 6$ . Metaphase showing one pair of chromosomes with secondary (nucleolar) constrictions. (s.c.) Fig. 2. Telophase showing two nucleoli. Fig. 3. Prophase showing chromosomes attached to the nucleolus at the secondary constrictions. Fig. 4. *C. zonatus*,  $2n = 8$ . Metaphase showing one pair of heteromorphic satellites (s). Fig. 5. Telophase showing two nucleoli of unequal sizes. Fig. 6. Prophase showing one pair of SAT-chromosomes attached to the nucleolus. Fig. 7. *C. aerius* var. *Gray Lady*,  $2n = 8$ . Metaphase showing one pair of heteromorphic SAT-chromosomes (A) and two other SAT-chromosomes (B, C) of different length and having satellites of unequal size. Fig. 8. Telophase showing four nucleoli of different sizes. Fig. 9. *C. ochroleucus*,  $2n = 10$ . Metaphase showing one pair of heteromorphic satellites (s). Fig. 10. Telophase showing two nucleoli of unequal size. Fig. 11. Prophase showing two SAT-chromosomes attached to the nucleolus. Fig. 12. *C. susianus*,  $2n = 12$ . Metaphase showing one pair of heteromorphic satellites (s). Fig. 13. Telophase showing two nucleoli of unequal sizes. Fig. 14. Prophase showing two SAT-chromosomes attached to the nucleolus. Fig. 15. *C. pulchellus*,  $2n = 12$ . Metaphase showing two pairs of satellites of two different sizes ( $s_1$ ,  $s_2$ ). Fig. 16. Telophase showing four nucleoli in one nucleus. Fig. 17. Prophase showing four SAT-chromosomes attached to the nucleolus.

9. *C. Tomasinianus* ( $2n = 16$ , Fig. 24)  $4\text{ Lst} + 4\text{ Mm} + 4\text{ Msm} + 2\text{ Ssm} + 2\text{ Sst}$ . Two satellites are present on the short arm of a pair of long chromosomes.

10. *C. Korolkowii* ( $2n = 20$ , Fig. 27),  $4\text{ Lst} + 8\text{ Msm} + 8\text{ Sst}$ . Four satellites are present, two on the short arm of a pair of medium chromosomes and the other two on the short arm of a pair of small chromosomes.

11. *C. Salzmännii* ( $2n = 24$ , Fig. 30)  $6\text{ Lst} + 2\text{ Msm} + 6\text{ Mst} + 6\text{ Sm} + 4\text{ Sst}$ . Two satellites and two secondary constrictions on the long arm of two pairs of long chromosomes.

12. *C. sativus* ( $2n = 24 = 3b$ , Figs. 33, 34)  $6\text{ Lst} + 3\text{ Msm} + 3\text{ Mst} + 3\text{ Ssm} + 9\text{ Sm}$ . Three satellites are present on the long arm of three long chromosomes.

13. *C. sativus* var. *Elwesii* ( $2n - 1 = 15$ , Fig. 41)  $4\text{ Lst} + 8\text{ Msm} + 3\text{ Sst}$ . Two satellites are present, one on the long arm of a long chromosome and the other on the short arm of a medium chromosome.

14. *C. Tournefortii* ( $2n = 30$ , Fig. 47). Constrictions cannot be properly located, due to the smallness and swelling of the chromosomes. Two satellites are present on the long arm of a pair of long chromosomes.

Regarding the morphology of the chromosomes, the following characteristic features may be noted: (a) There is a great range of variations in the size of the chromosomes possessing median, submedian, and subterminal constrictions. (b) Except in *C. Olivieri*, *C. ochroleucus*, and two satellites in *C. Korolkowii*, where satellites have been figured by Mather (1932), all other satellites have been found for the first time. (c) Out of all the species examined only two species with secondary constrictions were observed. This indicates clearly the predominance of satellites, as can also be inferred from the results of Heitz (1931a, b), Resende (1937), and Sato (1937). (d) From Table I, p. 231, it is evident that the number of satellites varies from two to six in different species. (e) There is a great range of variation in the size of the satellites, the largest being observed in *C. zonatus* (Fig. 4) and smallest in *C. aeriis* var. *Gray Lady* and *C. pulchellus* (Figs. 7, 15). There is a constant difference in size of the satellites of the same pair in *C. zonatus*, *C. ochroleucus*, and *C. susianus* (Figs. 4, 9, 12, respectively), or in the different pairs of satellites when more than one pair is present in the species, as in *C. aeriis* var. *Gray Lady*, *C. pulchellus*, *C. speciosus*, var. *albus* and *C. speciosus* (Figs. 7, 15, 18, 21 respectively).

Instances of the translocation of satellites may be observed in (i) *C. speciosus* var. *albus* (Fig. 18), where one satellite is present on the long arm and the other on the short arm of a pair of medium chromosomes, and in (ii) *C. aeriis* var. *Gray Lady* (Fig. 7), where four pairs of chromosomes are present but there is one satellite on the short arm of a small chromosome and another on the long arm of a long chromosome. This appears to be due to translocation, but it cannot be definitely stated which satellite has been really transferred. Instances of the translocation of satellites have been

reported in *Crepis tectorum* (Navashin, 1930, 1931), in seven species of *Crepis*—*C. biennis*, *C. ciliata*, *C. pulchra*, *C. artificiales*, *C. neglecta*, *C. bungei*, and *C. sibirica* (Swezy, 1935)—in species of *Aloe* (Resende, 1937b), in cultivated forms of *Paeonia albiflora* (Sinoto, 1938) and many others.

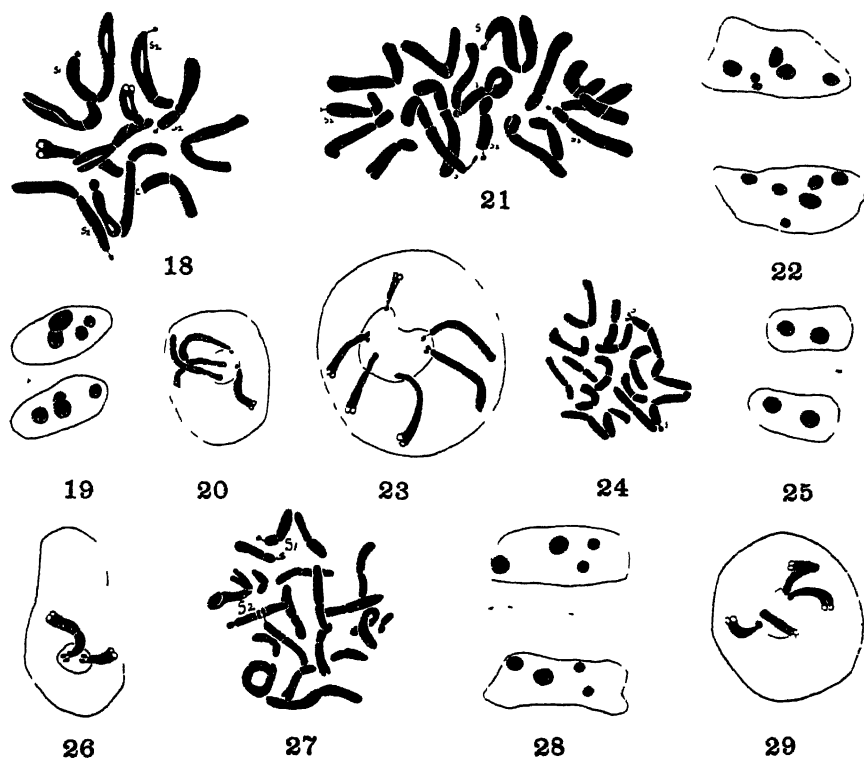
Fusions of the chromosomes have probably taken place in many species, but *C. ochroleucus* (Fig. 9) affords a very clear example. The complement consists of four pairs of chromosomes with subterminal constrictions and a fifth, which is the largest pair, has submedian constrictions. This pair seems to have been formed by fusion of two pairs of chromosomes with subterminal constrictions, and thus this species may be said to have been derived from a species with twelve chromosomes all having subterminal constrictions. Very clear cases of fusion have been shown in *Lycoris* (Inariyama, 1937) and in *Drosophila* (Bridges, 1919).

The heteromorphism in the size of the satellites and the great range of variation in the size of chromosomes in a complement indicate that the higher numbers may have been derived by extreme hybridization accompanied by translocation and fragmentation of the chromosomes.

#### *Organisation of nucleoli.*

In *C. sativus* Gates and Pathak (1938) have shown that each nucleolus arises as two bodies close together, one on each of the twin satellite threads of a telophase chromosome, showing conclusively the double structure of the chromosomes at this stage. As these nucleolar rudiments grow, they shortly fuse into one globular body which then grows to the full-sized nucleolus of the resting nucleus. In nine species mentioned against the serial numbers 1, 6, 7, 10, 11, 12, 13, and 14 in Table I, after using the differential stain with Feulgen and Light Green it was possible to see small green nucleolar droplets or granules of different size and shape in the early telophase. A representative example is given in *C. sativus* (Fig. 35). These granules may be simply aggregations of matrix substance, as Marshak (1931), by his microchemical studies, established a close relationship between the nucleolar substance and the matrix of the chromosomes. The different sizes of these bodies indicate that they fuse with each other. Later on they seem to have been collected on the satellite thread, probably due to the activity of the nucleolar body or organizer and partly of the satellites also. Thus by the end of the telophase nucleoli corresponding to the number of satellites or secondary constrictions are formed. They may fuse in many cases quite early. Only in rare cases were these bodies observed in the resting nucleus. That the nucleoli have a close connexion with the satellites is evident from their close association in prophase nuclei. Thus Table I, p. 231, shows that in all the species and varieties examined, the number of nucleoli in telophase corresponds exactly with those of the satellites and secondary constrictions and also an equal number of attachments of satellites to nucleoli in prophase.

The size of the nucleoli was observed to vary with that of the satellites. In



FIGS. 18-29. *Somatic figures*. (For magnification refer to description of Figs. 1-17.) Fig. 18. *C. speciosus*, var. *albus*,  $2n = 12 = 4b$ . Metaphase showing two pairs of satellites of two different sizes ( $s_1, s_2$ ). (At *c* one chromosome is cut but it is a homologue of the chromosomes lying at 3 o'clock.) Fig. 19. Telophase showing four nucleoli of two different sizes. Fig. 20. Prophase showing four SAT-chromosomes attached to the nucleolus. Fig. 21. *C. speciosus*,  $2n = 18 = 6b$ . Metaphase showing three pairs of satellites of two different sizes ( $s_1, s_2, s_3$ ). Fig. 22. Telophase showing six nucleoli of two different sizes. Fig. 23. Prophase showing six SAT-chromosomes attached to the nucleolus. Fig. 24. *C. Tomasinianus*,  $2n = 16$ . Metaphase showing a pair of satellites (*s*). Fig. 25. Telophase showing two nucleoli. Fig. 26. Prophase showing two SAT-chromosomes attached to the nucleolus. Fig. 27. *C. Korolkowii*,  $2n = 20$ . Metaphase showing two pairs of satellites of two different sizes ( $s_1, s_2$ ). Fig. 28. Telophase four nucleoli of two different sizes. Fig. 29. Prophase showing four SAT-chromosomes attached to the nucleolus.

*C. Tomasinianus* and *C. Tournefortii* (Figs. 24, 47), where the satellites in a pair are of equal size, the nucleoli are also similar. Where the size of the satellites has been found to differ in the same pair, as in *C. zonatus*, *C. ochroleucus*, *C. susianus*, and *C. sativus* var. *Elwesii* (Figs. 4, 9, 12, 41) or in different pairs when more than one pair is present in the species, as in *C. speciosus* var. *albus*, *C. speciosus*, *C. pulchellus*, and *C. aeneus* var. *Gray Lady* (Figs. 18, 21, 15, 7), the nucleoli differ correspondingly in size. In *C. Salzmannii*, possessing one pair of satellites and one pair of secondary constrictions, the nucleoli corresponding to the satellites are larger than those



corresponding to the secondary constrictions (Fig. 31). The satellites and secondary constrictions are present on the long arm of the long pair of chromosomes. But the secondary constrictions are included in the nuclei a little earlier than the satellites because the satellites are at the ends of finger-like projections of the nuclear membrane. The constrictions are therefore situated in the nuclei more towards the poles than are the satellites. This example also shows that priority of being included in the nucleus does not mean that the resulting nucleus will be larger. In addition to the time factor there seem to be other factors (e.g. length of the thread) which determine the size of the nucleolus. This subject will be discussed later.

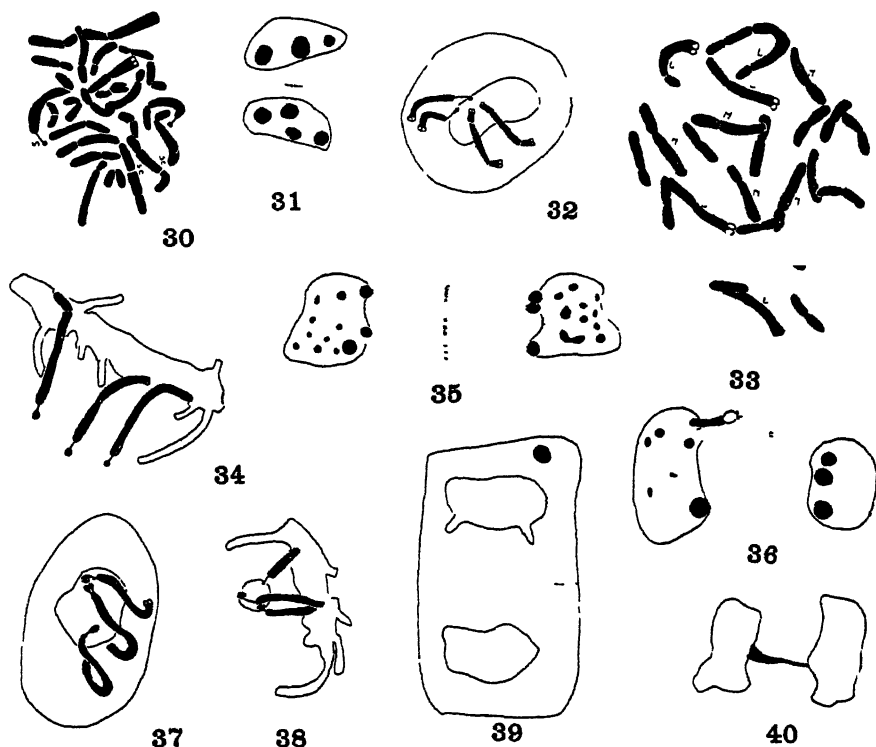
#### *The behaviour and function of the nucleoli.*

That nucleoli appear in telophase and disappear by late prophase has long been a matter of common observation. But enough instances are now known where investigators have observed their persistence at metaphase as in *Zea* (Zirkle, 1928), *Cucurbita* (Frew and Bowen 1929), *Brassica* (Catchside, 1934), *Callisia* (Derman, 1933), and many others.

In almost all the species of *Crocus* examined nucleoli have been observed to persist at least up to metaphase. A representative case is given in *C. sativus* (Fig. 38) where the three satellites are still attached to the single nucleolus lying on one side of the metaphase (side view). One, two or all the three nucleoli may persist, and as they are attached to the satellites which are present on the long arm of the long chromosomes, they remain away from the plate towards any pole. If they still persist, they move towards the pole nearest which they were situated at metaphase, and at telophase they are thrown outside the spindle (Fig. 39). Later these nucleoli are included in the cytoplasm, but they may persist and be observed even in the prophase cells. Such persistence of nucleoli may be due to the quick succession of divisions in the meristematic cells.

The close association of chromosomes and nucleoli from telophase to prophase, and their disappearance at late prophase, indicates that the nucleolar materials, completely or partly, may flow back again to the chromosomes, as suggested by Van Camp (1924), Sheffield (1927), Zirkle (1928, 1930), Perry (1932), and Gates (1937). But the persistence of nucleoli to metaphase in almost all the *Crocus* species suggests that all the nucleolar material is not needed by the chromosomes, if it is taken back at all.

Various functions have been attributed to the nucleolus in text-books of cytology, as in Sharp (1934) and Wilson (1925). By the heterogeneity of these it may be inferred that the function of the nucleolus is still a mystery. But the regular appearance and disappearance indicates that it has something important to do in the economy of the cell. Probably during the process of formation of the relatively simple substance of the nucleolus from chromatin, which is a very complex substance, much energy may be released which is utilized in the metabolism of the cell.



FIGS. 30-40. *Somatic figures*. (For magnification see description to Figs. 1-17.) Fig. 30. *C. Salzmannii*,  $2n = 24$ . Metaphase showing one pair of satellites (s) and one pair of secondary (nucleolar) constrictions (s.c.). Fig. 31. Telophase showing four nucleoli of two different sizes. Fig. 32. Prophase showing two chromosomes with satellites and two chromosomes with secondary constrictions attached to the nucleolus. Fig. 33. *C. sativus*,  $3n = 24$ . Metaphase plate (polar view). Large chromosomes are marked by 'L' and medium by 'M', and the rest are short chromosomes. Fig. 34. Metaphase plate (side view) showing three SAT-chromosomes. Fig. 35. Telophase showing origin of nucleoli as droplets which may fuse with each other before being organized or collected at the satellite thread. Fig. 36. Later telophase showing one nucleus with one protruding chromosome, which seems to be a little dragged by the action of microtome knife and shows very well two satellite knobs and collection of the nucleolus in the form of a collar round the satellite thread. In the other nucleus three nucleoli have been formed. Fig. 37. Prophase showing three SAT-chromosomes attached to the nucleolus. Fig. 38. Metaphase (side view) showing persisting nucleolus to which all the SAT-chromosomes are still attached. The knob in one satellite is not clear. Fig. 39. Telophase showing one persisting nucleolus. Fig. 40. Somatic bridge.

### *Polyploidy and basic numbers in the genus.*

The presence of a haploid number as low as three has been reported only in four genera, *Callitriche autumnalis* (Jorgensen, 1923), *Crepis capillaris* (Babcock and Navashin, 1930), *Zachyntha verrucosa* (Navashin, 1930) and four species of *Crocus* (Mather, 1932) and one *Crocus* species (Karasawa, 1939). With such a low haploid number it is quite possible that plants having comparatively very low chromosome numbers may be polyploid.

Heitz (1926), after determining the chromosome numbers of six species, concluded that six may be the basic number, although all the chromosome numbers found by him are not exactly in the series. Mather (1932) found very little direct evidence of polyploidy. In *C. vernus* with varieties having eight, eighteen, and nineteen chromosomes, he suspected the presence of a series, but it does not appear to be a clear case. He also suggested polyploidy in the varieties of *C. speciosus* with twelve and eighteen chromosomes. Karasawa (1932) ascertained a polyploid series in the varieties of *C. vernus* having  $2n$ ,  $3n$ , and  $4n$  chromosomes, with eight as basic number. In 1933 he found *C. sativus* to be autotriploid on the basis of the formation of eight trivalents at the metaphase of heterotypic division. In 1935 he traced another polyploid series in the varieties of undetermined species with twenty and thirty chromosomes, with ten as basic number. At the same time, he suggested that *C. susianus*, having fifteen chromosomes and forming trivalents at metaphase I in meiosis, is an autotriploid probably with five as basic number.

For establishing a polyploid series where there are different basic numbers the study of the chromosome morphology along with the relation of nucleoli to satellites, which has been practically neglected in the above-mentioned cases, is absolutely necessary, as the number of chromosomes alone cannot be the final criterion. A glance at Table I, p. 231, shows that the number of satellites and nucleoli ranges from two to six. On the hypothesis that a haploid set contains usually one satellite and one nucleolus, the presence of diploid triploid, tetraploid, and hexaploid plants is expected in the species examined.

A very interesting case was observed in *C. speciosus* var. *albus* and *C. speciosus* (Figs. 18, 21), the former having twelve chromosomes with four satellites, four nucleoli in telophase, and four SAT-chromosomes attached to nucleolus in prophase (Figs. 19, 20) the latter having eighteen chromosomes with six satellites, six nucleoli at telophase and six SAT-chromosomes attached to the nucleolus in prophase (Figs. 22, 23). The variation in satellite size has already been described on p. 233. The evidence indicates that the 'variety' of *C. speciosus* is a tetraploid and the species itself hexaploid. Obviously the 'variety' cannot have been derived from the 'species'. A comparison of their chromosome morphology, particularly of the SAT-chromosomes, reveals at once how the two must have originated. The SAT-chromosomes are represented in Diagram I.

It is suggested that the tetraploid has probably been derived by doubling the chromosomes of a diploid with six chromosomes and possessing two satellites, one on the short arm of a long chromosome and the other on the long arm of a medium chromosome, although a diploid of this constitution is not present in any species examined to date. In the tetraploid, one satellite from the long arm of the medium chromosome is translocated to the short arm of the same chromosome. The hexaploid species must have been derived by a cross between the tetraploid and a diploid species, giving rise to a sterile triploid with nine chromosomes, which by doubling gave rise to the allopolyploid.

ploid species with eighteen chromosomes. Karasawa (1937) in *C. speciosus* observed the formation of usually nine bivalents which may also be present in an allohexaploid. The following scheme (Diagram 2) explains the derivation of the SAT-chromosomes in the hexaploid species.

From this scheme it is evident that there are two long and two medium SAT-chromosomes in the tetraploid, while in the hexaploid the number of long SAT-chromosomes remains the same as in the tetraploid but the medium SAT-chromosomes have been doubled. This is found to be true in the case of the other long and medium chromosomes, as is apparent when the chromosome morphology of the two species is compared (p. 231).

The above-mentioned facts definitely prove the presence of polyploidy in the species, which was barely suggested by Mather (1932) on the basis of the chromosome numbers.

*C. sativus* (Fig. 33) has been suggested by Karasawa (1933) to be an autotriploid. This is strongly supported by the chromosome morphology, where sets of three homologous chromosomes may be recognized, with three satellites, three nucleoli in telophase, and three satellites attached to the nucleolus in prophase (Figs. 34, 36, 37).

*C. sativus* var. *Elwesii* (Fig. 41), having an odd number of fifteen chromosomes, is suggested by Mather to be a hybrid which remained as such without doubling its complement. The species possesses two satellites of unequal size, the larger one on the long arm of a long chromosome and the smaller one on the short arm of a medium chromosome. The inequality in the size of

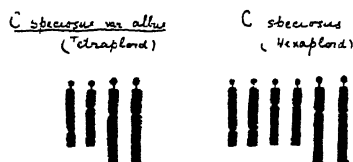


DIAGRAM 1

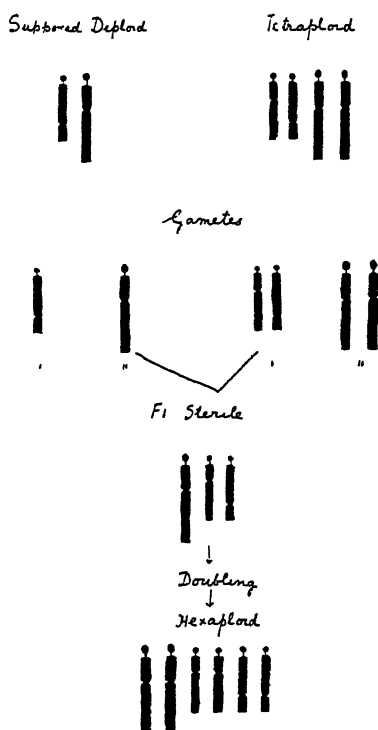


DIAGRAM 2

the satellites present on non-homologous chromosomes, and a corresponding variation in the size of the nucleoli (Fig. 42), indicate that this species may be a hybrid. It may have been derived by a cross between two diploid species (probably having sixteen chromosomes), one having a pair of large satellites and the other a pair of small ones of the description given above. The

number fifteen is accounted for by the loss of one chromosome, as was also suggested by Karasawa (1937). *C. sativus*, which has all the three satellites on the long arm of the long chromosomes, may have been derived from the assumed diploid species with two large satellites on the long arm of long chromosomes, by the union of a reduced and an unreduced gamete.

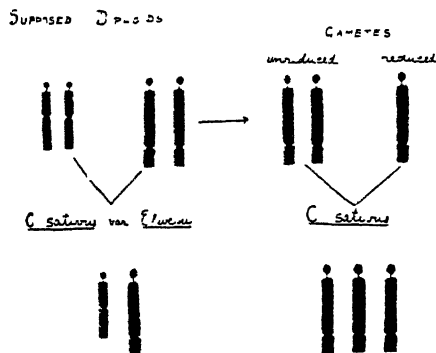


DIAGRAM 3

The following scheme (Diagram 3), dealing with the SAT-chromosomes, may be illustrative.

*C. pulchellus* ( $2n = 12$ , Fig. 15) would be taken as a diploid on the basis of six, as Heitz (1926) suggested. But the presence of four satellites, four nucleoli in telophase and four attachments of satellites to nucleolus in prophase (Figs. 16, 17) indicates that it may be tetraploid. The presence of two sizes of satellites and nucleoli further suggests the hybrid nature of the species. *C. ochroleucus*, *C. Korolkowii*, and *C. Tournetfortii* (Table I, p. 231) have ten, twenty, and thirty chromosomes, respectively. From the number alone it can easily be said that they form an ideal polyploid series. But their chromosome morphology and the number of satellites and nucleoli in telophase indicate that they are not in the same series.

All the species mentioned in Table I possessing two satellites and two nucleoli in telophase may be regarded as diploids. Thus it appears that in the species examined diploid, triploid, tetraploid, and hexaploid species are present.

From the presence of different basic numbers, five, six, eight, and ten, it appears that polyploid series may have developed on different lines. To find out the original basic number may require exact study of many more species. But the polyploid series established here in the varieties of *C. speciosus* definitely shows that the basic number must be three. This number, which corresponds to the lowest haploid number, may be taken as the original basic number. All other numbers may have been derived from this or may have a different origin.

### *Somatic doubling.*

*C. sativus* var. *Elwesii* has already been described as possessing fifteen chromosomes, two satellites, and two nucleoli in telophase. In tetraploid cells along with the doubling of the chromosome numbers, including the SAT-chromosomes, the nucleoli in telophase were also observed to be doubled (Figs. 45, 46). The size of the root-tips as observed in the cross-sections

increased with the increase of tetraploid cells in them. In this way three types of roots have been distinguished: (i) having cells with only fifteen chromosomes, (ii) having cells with both fifteen and thirty chromosomes in different proportions, (iii) having cells mostly with thirty chromosomes. In the second type of roots excellent examples of sectorial chimaeras may be observed. In photomicrograph (Fig. 62) a demarcation line separates the sector with the tetraploid cells from the normal cells. From a comparison of the two, it will be obvious that with the doubling of the chromosomes an increase in size of the cells and nucleoli has taken place. The volume of the nucleoli (Table II) was found to be approximately doubled. This confirms de Mol's idea that the volume of nucleolus increases with the increase in chromosome numbers or with polyploidy.

TABLE II

*C. sativus* var. *Elwesii*. Nucleoli were Measured from Ten Inner Cortical Cells.

Volume of Nucleoli		Approximate ratio.
Normal cells.	Tetraploid cells.	
51521.4 units	99067.8 units	1 : 2

From the tetraploid sectors buds may arise in the corms, producing plants with the doubled number.

Karasawa (1937) observed at meiosis seven bivalents and one univalent. He found the percentage of sterility in the pollen-grains very high and attributed it mostly to the erratic behaviour of the univalent.

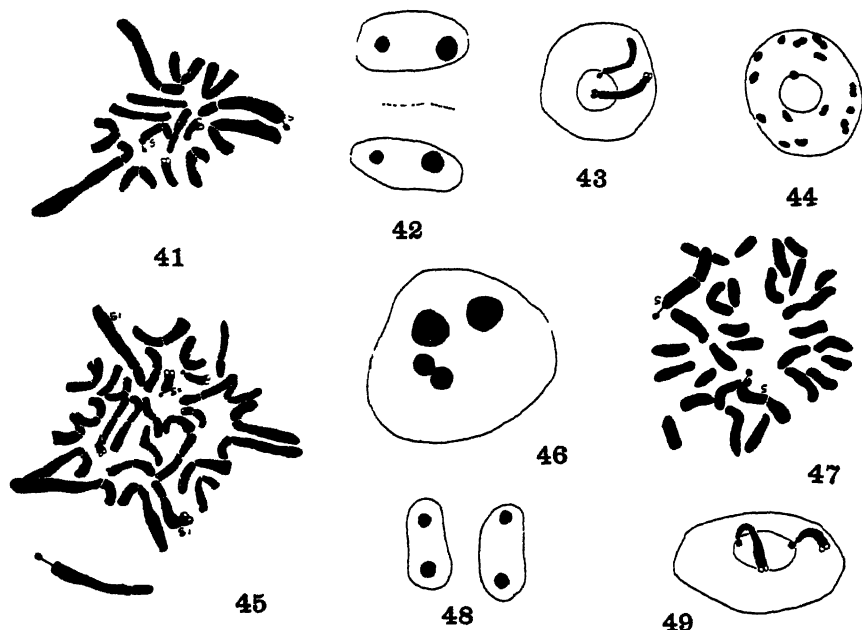
The presence of a large number of tetraploid cells indicates that the species might, by doubling its chromosome number, develop a more stable and fertile form.

In *C. Olivieri* ( $2n = 6$ ) the doubling could be judged only from the side view of metaphase chromosomes. Nuclei with four nucleoli, as against two in the species, were also observed. The occurrence of tetraploid cells in *C. speciosus* ( $2n = 18$ ) and *C. Sieberi* ( $2n = 22$ ) has been reported by Karasawa (1937) and in *C. biflorus* ( $2n = 8$ ) by Mather (1932). The latter has also figured the doubling of the SAT-chromosomes. Somatic doubling is now a recognized phenomenon both in plants and animals. A number of plants have been reported showing this phenomenon, as in *Tragopogon pratensis* (Winge, 1917), *Spinacia oleracea* (de Litardière, 1923), *Cannabis sativa* (de Litardière, 1925, and Breslawetz, 1926), *Butomus umbellatus* (Terby, 1924), *Solanum lycopersicum* (Lesley, 1925), *Brassica oleracea* and *B. montana* (Netroufal, 1927), *Crepis* (Navashin, 1926, and Hollingshead, 1930), *Cicer arietinum* (Milovidov, 1932, and Iyengar, 1939), *Gasteria cheilophylla* and *G. brevifolia* (Sato, 1937), *Paeonia albiflora* (Sinoto, 1938), and others.

Recently Srinath (1939) has discussed the significance and the causes of somatic doubling.

*Prochromosomes.*

The credit of reporting prochromosomes or chromocentres as deep-staining bodies present in the resting nucleus goes to Rosen (1892), but their definite correspondence with the number of chromosomes in a particular species was



FIGS. 41-9. *Somatic figures.* (For magnification see description of Figs. 1-17.) Fig. 41. *C. sativus* var. *Elwesii*,  $2n = 15$ . Metaphase showing two heteromorphic SAT-chromosomes ( $s$ ). Fig. 42. Telophase showing two nucleoli of unequal sizes. Fig. 43. Prophase showing two SAT-chromosomes attached to the nucleolus. Fig. 44. Resting nucleus showing fifteen pro-chromosomes. Fig. 45. Metaphase showing doubled number of chromosomes with two pairs of heteromorphic SAT-chromosomes ( $s_1, s_2$ ). Satellites on the long SAT-chromosomes ( $s_1$ ) are not present in this plate. One such chromosome with satellite has been sketched separately, from another plate. Fig. 46. Resting cell showing four nucleoli. Fig. 47. *C. Tournefortii*,  $2n = 30$ . Metaphase showing one pair of SAT-chromosomes ( $S$ ). Fig. 48. Telophase showing two nucleoli. Fig. 49. Prophase showing two SAT-chromosomes attached to the nucleolus.

shown by Rosenberg (1904, 1909). Recently Doutreligne (1933), Smith, (1934) in *Impatiens*, Manton (1935) in *Biscutella laevigata*, and Raghaven (1938) in *Polanisia trachysperma* and *Gynandropsis pentaphylla* have shown that these bodies consist of the proximal part of the chromosomes on either side of the primary constriction.

All the above-mentioned plants have very small chromosomes. But after studying *Crocus* species with preparations specially stained in Feulgen, pro-chromosomes were found in plants with fairly long chromosomes, as in *C. zonatus*, *C. pulchellus*, *C. speciosus* var. *albus*, *C. Salzmännii*, *C. sativus*, and *C. sativus* var. *Elwesii*. They correspond usually with the number of the

chromosomes in the species. A representative example has been given for *C. sativus* var. *Elwesii* (Fig. 44).

#### *Somatic bridge.*

In root-tip cells of *C. sativus* four cells showed bridge formation by chromosomes at anaphase or very early telophase. No fragment was observed in any case. Fig. 40 shows a bridge which is thick at one end and thin at the other. The thin portion appears to be under tension from stretching. The bridge may break at any point, and thus a change in the chromosome balance may be caused, resulting in unequal distribution of the chromatin in the two nuclei.

The cause of bridge formation may be that the ends of the chromatids after separation may fuse again, as has been suggested by Barber (1938) in *Paeonia Veitchii* and *Kniphofia rufa*, though this goes against the well-established idea that the chromosome ends are usually unable to fuse without fragmentation. Husted (1936) and Riley (1936) have shown the occurrence of somatic bridges after irradiation.

#### B. Meiotic

##### *C. susianus.*

In this species ( $2n = 12$ ) usually six bivalents are formed at diakinesis (Fig. 51) and at metaphase I (Fig. 53); but in some cases a ring of four chromosomes along with four bivalents were found at both these stages (Figs. 52, 54). At diakinesis one bivalent was always found attached to the fused nucleolus, confirming the presence of a pair of SAT-chromosomes in somatic cells. At a little earlier stage than diakinesis the two satellites are seen quite clearly (Fig. 50). At later stages they become fused and are included in the body of the contracting chromosomes and hence cannot be easily made out. (Later stages were not available in this material.)

Cleland (1922) first found a ring of four chromosomes in *Oenothera*, but it was Belling (1925) who first suggested that ring formation is due to interchange of segments between non-homologous chromosomes. Later, chromosome rings have been reported in many plants and animals. The cases observed in diploids may be grouped into the following three classes: (1) In hybrids obtained by crossing different races, as in *Datura* (Blakeslee, 1928, 1929), *Pisum* (Hakansson, 1929, 1931, 1934; Sansome, 1929, Pellew and Sansome, 1931), *Polemonium* (Clausen, 1931), *Oenothera* (Gates and Catcheside, 1932), and *Triticum* (Smith, 1936). (2) After irradiation, as in *Zea Mays* (McClintock, 1931), *Drosophila* (Muller, 1930), *Triticum* (Katayama, 1935), *Oenothera* (Catcheside, 1935), and *Oryza* (Ramiah, Parthasarathy and Ramanujam, 1934; Parthasarathy, 1939). (3) In naturally occurring forms, as in *Oenothera* (Cleland, 1922, see Gates and Ford, 1938), *Campanula* (Gardiner and Darlington, 1931), *Humulus* (Kihara, 1929 and Sinoto, 1929), *Rhoeo* (Belling, 1927, and others), and *Crocus chrysanthus* (Darlington, 1937). The exact mode of origin in these forms is still a matter of speculation. The



stability of the heterozygotes is due to the elimination of the homozygous forms, as in *Oenothera*.

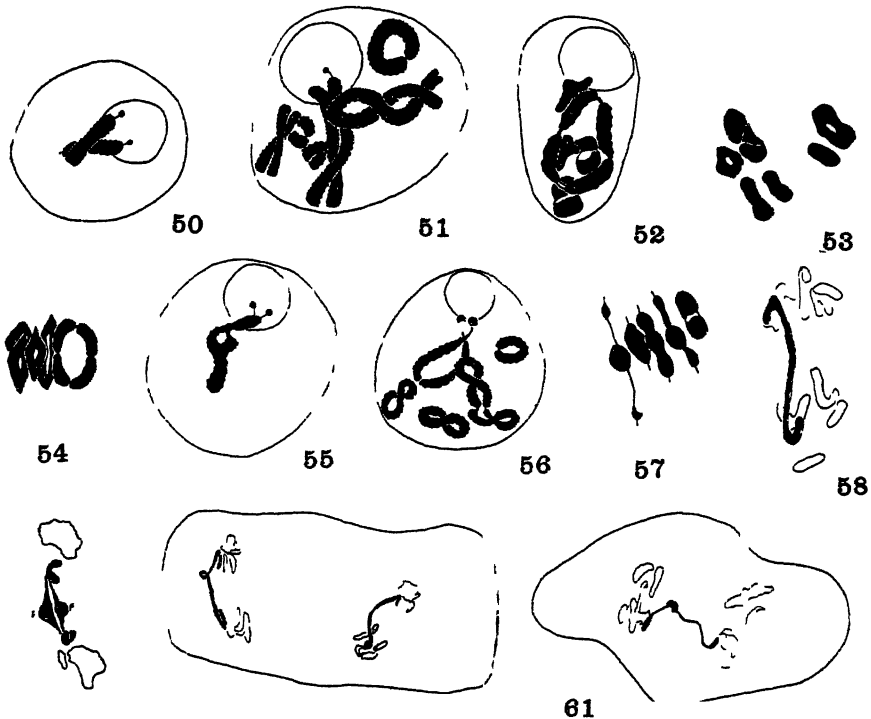
The formation of a ring of four chromosomes in all the above-mentioned instances may be explained on the segmental interchange hypothesis suggested by Belling. A different interpretation may be required in *C. susianus*, which, as suggested before, may be a secondarily balanced diploid. A pair of chromosomes has been reduplicated to give rise to four homologous chromosomes which should behave at pachytene just like those observed in a tetraploid. It has been shown in tetraploid *Allium* (Levan, 1935) that the chromosomes come together in pairs. If it is assumed that different blocks in different chromosomes behave differently, a single chromosome can pair at more than one point with different chromosomes. In this way if chiasmata are formed favourably, a ring of four chromosomes may be formed and by the failure of any chiasma a chain of four chromosomes will result. If the four chromosomes pair separately two bivalents will be formed.

#### *C. ochroleucus*.

In this species ( $2n = 10$ ) five bivalents are formed at metaphase I (Fig. 57). At diakinesis (Fig. 56) one bivalent was always found attached to the nucleolus, confirming the presence of a pair of satellites in somatic cells. Satellites can be properly seen at a little earlier stage than diakinesis (Fig. 55). Karasawa (1937) studied the meiosis of this species and also found five bivalents at first division. He observed irregularities like lagging of chromosomes, unequal distribution, and sometimes even the scattered nature of the chromosomes showing the laxity in the meiotic process.

Besides the irregularities mentioned above, another type was observed for the first time in this species. The formation of chromatid bridges was observed at both divisions I and II. The initial cause of the formation of chromatid bridges is the presence of inverted segments in chromosomes where different types of cross-overs can take place. Their presence in chromosomes can be detected at pachytene when normal pairing is disturbed and at anaphase I and II when dicentric chromatid bridges and acentric fragments result. At anaphase I a single dicentric bridge and an acentric fragment (Fig. 58) is formed where there is only one cross-over in the inversion; and double dicentric bridges and acentric fragments (Fig. 59) are formed when there are two complementary cross-overs in the inversion. When there is one cross-over in the inversion and another proximal to it, i.e. between the inversion and the centromere, in the same chromatid, a monocentric loop and a fragment result in the anaphase I. This loop, after the division of the centromere, forms a dicentric bridge at anaphase II (Fig. 60), where a bridge may be formed in only one diad or both depending upon the number of chromatid loops formed at anaphase I. In the figure mentioned above one bridge is present in each diad and no fragment.

By the end of anaphase or telophase I the bridge undergoes extreme stretch-



FIGS. 50-61. *Somatic figures*. (For magnification see description of Figs. 1-17.) Fig. 50. *C. susianus*,  $2n = 12$ . Part of one bivalent at late diplotene, having two satellites, attached to the nucleolus. Fig. 51. Diakinesis showing six bivalents, one of which is attached to the nucleolus. Fig. 52. Diakinesis showing a ring of four chromosomes and four bivalents. Fig. 53. Metaphase I (polar view) showing six bivalents. Fig. 54. Metaphase I (side view) showing a ring of four chromosomes, and four bivalents. Fig. 55. *C. ochroleucus*,  $2n = 10$ . Late diplotene showing one bivalent, having two satellites, attached to the nucleolus. Fig. 56. Diakinesis showing five bivalents, one of which is attached to the nucleolus. Fig. 57. Metaphase I showing five bivalents. Fig. 58. Anaphase I showing one chromatid bridge with no fragment. Fig. 59. Anaphase I showing one double chromatid with the two fragments (F) still attached. Fig. 60. Anaphase II showing one chromatid bridge in each diad. Fig. 61. Anaphase II showing one persisting bridge and one nucleolus.

ing and it may break at any weak point. But in some extreme cases where the bridge is formed by long chromosomes, it may persist in anaphase II also (Fig. 61).

Fragments are formed by the distal portions of the chromosomes. A fragment may be present in the anaphase I as a free body or attached to the chromatid (Fig. 59) or may be absent (Fig. 58). This shows that they may be lost at any stage. The size of a fragment is inversely proportional to that of the inverted segment and hence the size of a fragment may give a fair measure of the length of the inversion.

The occurrence of inversion bridges has been reported in pure species, as in *Trillium* (Smith, 1935), *Tulipa* (Upcott, 1937), *Chorthippus*, *Stauroderus*

(Darlington, 1936), *Paeonia* (Sax, 1937); in hybrids, as in the species of *Crepis* (Müntzing, 1934), *Triticum* (Mather, 1935), *Lilium* (Richardson, 1936), *Nicotiana* (Müntzing, 1935), and others. They have been found after irradiation in *Zea Mays* (McClintock, 1933) and *Drosophila* (Painter and Stone, 1935, and Gruneberg, 1935).

The significance of the formation of a bridge and a fragment was first inferred by McClintock (1933) as cytological evidence for crossing-over in the inverted segment. Inversions are now used to separate natural races in *Drosophila* (Sturtevant and Dobzhansky, 1936; Koller, 1935). The formation of such bridges may result in creating chromosome unbalance due to loss of the fragments and unequal division of the chromatid bridge. This unbalance may be responsible to some extent in causing disturbance in the fertility of the species.

## 5. DISCUSSION

### *Satellites and nucleoli and their relation to phylogeny.*

Since the discovery of a pair of satellites or trabants in *Galtonia candicans* by Navashin (1912), a mass of literature has appeared describing these structures in plants. Gates (1937, 1938) has reviewed the most relevant literature on the subject. The investigations done on a large scale showing these structures, as in 37 species of *Thalictrum* (Kuhn, 1928), 33 species of *Vicia*—26 with satellites and 7 with secondary constrictions (Heitz, 1931*b*)—48 species and 3 hybrids from *Aloinae* (Sato, 1937), 151 species of *Aloinae* and 34 species of different genera (Resende, 1937), and 4 species of *Paeonia* with about 80 forms of *P. albiflora* (Sinoto, 1938), &c., have given ample evidence as regards their presence in plants as permanent components of some chromosomes.

The present conception of a satellite is a thread and a knob or head, both chromatic in nature. The chromatic nature of the knob has been described by almost all the investigators, but till 1937 the thread was regarded as achromatic in nature. The credit of showing this goes to Fernandes (1937), who found that the thread does give Feulgen reaction. Heitz (1931*b*), showed that the nature of the thread in the secondary constriction is similar to the satellite thread. Fernandes (1936) even distinguishes two types of satellites (i) heterochromatic, and (ii) euchromatic. The former only remains usually attached to the nucleolus during telophase, interphase and prophase. In all the species of *Crocus* examined the thread was stained with Feulgen. Polymorphism with regard to variation in the size of the satellites and their presence or absence in plants has been described.

Taylor (1925 *b, c*) showed the presence of SAT-chromosomes in *Crepis setosa*, *C. capillaris*, *Aloe saponaria*, and *Gasteria*. In 1926 he observed minor differences between the satellite sizes in a particular cell and attributed it to the action of fixatives. Emme (1925) reported in *Hordeum* polymorphism with regard to the presence or absence of one or both satellites of a pair.

Baranov (1926) found in *Drimiopsis maculata* ( $2n = 64$ ) four large and twelve to sixteen small satellites attached to long and short chromosomes respectively. Navashin (1927) in *Galtonia candicans* differentiated between symmetrical and asymmetrical races having similar and dissimilar size of

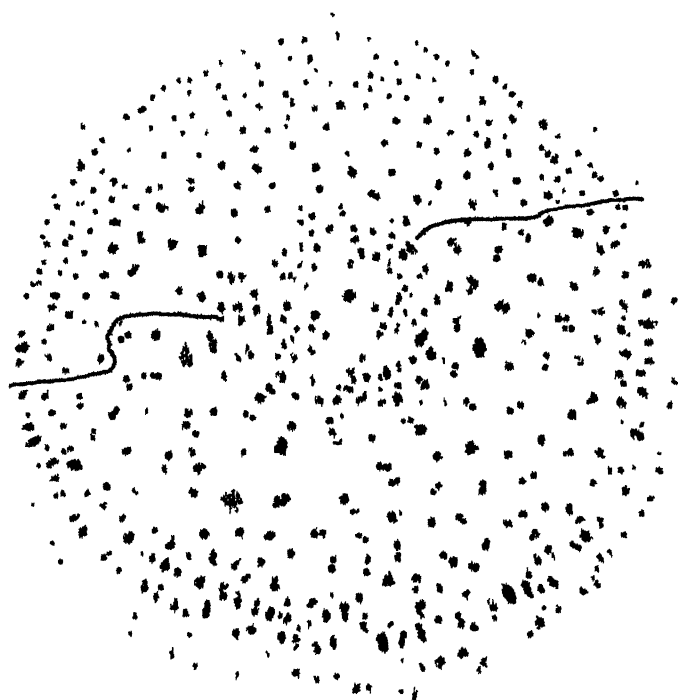


FIG. 62. *Crocus sativus* var. *Elwesii*,  $2n = 15$ . Microphotograph of T.S. root-tip showing normal sector above and tetraploid sector below.

satellites in somatic cells. Medwedewa (1929) differentiated three races of *Crepis dioscoridis*, one having large satellites, the other with small, and the third heterozygous. Navashin (1927) found the disappearance of satellites—amphiplasty—in some species-crosses in *Crepis*. He (1934) observed the same phenomena in thirteen more crosses and differentiated between differential amphiplasty, affecting individual chromosomes, and neutral amphiplasty, affecting the whole genome.

Fernandes (1935) has described in *Narcissus reflexus* and *N. triandrus* a great range of satellite size in the same individual, the largest being equal to the arm of a chromosome and the smallest represented merely by a thread, and that too was found to be absent in many cases. Sinoto (1938) in *Paeonia* found variation in the satellite sizes and thought it to be due to translocation of portions of satellites. Lesley (1939) found, in tomato, satellites of different

sizes and attributed their increase to addition of extra satellitic material. Men-sinkai (1939) thought that a satellite is a rolled end of the chromonema. He attributed the variation in size of the satellite to the rolling or unrolling of the satellite itself; by the former process it will appear bigger and by the latter, smaller.

In *Crocus* species satellites of different sizes have been observed. Minute changes in the size of a particular pair may be due to the action of fixatives, but constant difference in size of the same pair or of different pairs in a species may be due to the stage of evolution of the particular species in the genus, the most primitive ones possessing large, and advanced ones possessing minute, satellites.

### *Organization of nucleoli.*

Though in the past few years this subject has attracted the attention of many workers, yet the different views in the field are still definitely conflicting. The appearance of the nucleolus in telophase and its disappearance in late prophase has been observed long since, but the mode of organization was first discovered by Van Camp (1924) in *Clivia miniata* by fusion of small globules or droplets forming larger ones, in telophase. These bodies appeared to be attached to the 'continuous spireme'. A close resemblance between these nucleolar bodies or substances and the matrix of the chromosomes has been demonstrated through microchemical tests by Marshak (1931). But the first morphological relation between the chromosomes and the nucleolus was demonstrated by Latter (1926) in the pollen development of *Lathyrus odoratus*, where she found in prophase that the spireme was attached to the nucleolus by a deep-staining body which she called the 'nucleolar body'. This body was later observed in a number of plants, as in *Oenothera* (Sheffield, 1927; Gates and Sheffield, 1929), *Lathraea* (Gates and Latter, 1927), *Malva sylvestris* (Latter, 1932), and *Oryza* (Selim, 1930).

Heitz (1931a, b), after studying the somatic mitosis of a number of plants, came to the conclusion that the nucleoli arise on the thread (achromatic) of the satellites or secondary constrictions of nucleolar chromosomes. But later, due to his observation of three nucleoli in *Vicia Faba* and *V. monanthus* possessing only two SAT-chromosomes and due to the production of a nucleolus in a micronucleus from a single lagging chromosome without satellite, he modified his views, concluding that in the absence of a SAT-chromosome other chromosomes may give rise to nucleoli.

Derman (1933) observed a similar method of organization to that suggested by Van Camp and found that in *Callisia* the nucleolar number does not correspond to the number of satellites. He criticized Heitz's hypothesis, arguing that the satellite thread, where it is attached to the nucleolus, is always on the side or surface and the nucleolus does not form a collar which it ought to have done if it was organized on the thread.

McClintock (1934) in *Zea Mays* showed the organization of nucleolus due

to the activity of a deep-staining body at the end of the SAT-chromosomes both in somatic and meiotic cells. This body, which she called nucleolar organizing element, may be the same as the nucleolar body described by Latter (1926). McClintock observed in type IV microspore nucleolar granules along all the chromosomes in the absence of SAT-chromosomes. She attributed this to genomic deficiency. Woods (1937) observed a similar case in the microspores of a strain of *Tulipa*. Navashin (1934) found in species crosses of *Crepis* that the rate of the capacity of the organizer varied in different species.

Fernandes (1936) does not consider the nucleolar chromosomes and SAT-chromosomes to be synonymous, and he thinks that the filament of the satellite is not indispensable for the formation of a nucleolus. According to him, the position of the point of greatest activity of the nucleogenic region is not always the same, but varies with the shape of the satellite in the metaphase chromosomes.

Gates (1937) suggested that 'the nucleolar body appears to act normally as an organizing centre at one locus of a particular chromosome pair, apparently controlling the disposal of the matrix material of the chromosomes which is given up in telophase and may be, at least in part, distributed to the chromosomes again in the following prophase'. Later he (1938) thought that 'in the abnormal absence of this organizing body the matrix material from the various chromosomes simply accumulates in droplets in the nucleus'. Gates and Pathak (1938) have shown in *Crocus sativus* the origin of the nucleolus as two bodies, one on each of the twin satellite threads in telophase. They shortly fuse to form one globular body. Dearing (1934) has observed in *Amblystoma* a similar mode of organization of nucleolus.

From the mode of organization of nucleolus described here in *Crocus* species, it appears that besides the organizer satellites may also take a part in organization. This suggestion will also explain the formation of a large nucleolus by the smaller half of the organizer which was accompanied by the satellite (McClintock, 1934). Further, it will explain the other point, which was raised by Derman (1933) and McClintock as regards the position of the satellite thread on the surface of the nucleolus in prophase if it is attached at all. In telophase the nucleolus does form a collar (Fig. 36). Then again, assuming that the organizer alone forms the nucleolus and if the point of attachment in prophase is taken as a criterion for the locus of organization, it will be very difficult to explain the cases where the SAT-chromosomes are attached to the nucleolus only by the satellite knob. Such instances will evidently support the suggestion that along with the organizer, the satellite also takes part in organization of the nucleolus. This finds further support from the work of Lesley (1939) who found, in Tomato, that strains with a large satellite had a large nucleolus and those with a small satellite had a small nucleolus.

When the nucleoli are organized in telophase, their number corresponds with those of the satellites plus the secondary constrictions. This has been

shown by Heitz (1931a, b), McClintock (1934), Resende (1937), Sato (1937), Sinoto (1938), and many others. All the *Crocus* species studied here showed the same relation.

The question of the variation in the size of the nucleoli according to that of the satellites appears to be closely connected with the question of the organization of the nucleolus. Heitz (1931) concluded that the size of the nucleolus depends upon that of the thread. McClintock thought that the size may vary with the functioning capacity of the organizer. Navashin (1934) found no correlation between the two, while Sato (1937) and Resende (1937) found a correlation.

A more definite contribution in this connexion has been advanced by Lesley (1939) in tomato. She found three sizes of satellites: (i) on the 'short A' chromosome, (ii) on the 'long A' chromosome, and (iii) on 'very long A' chromosome. The size of the satellite in case of the second is double that of the first and that in the third is double that of the second. She attributes the difference in size to the addition of extra satellitic materials. The sizes of the nucleoli produced also vary according to those of the satellites. In *Crocus* also some correlation was observed between the two.

It is obvious now that normally the SAT-chromosomes are responsible for the organization of the nucleoli. The study of chromosomes in general and SAT-chromosomes in particular found a very practical application in their relation to phylogeny. But such a study requires an exact and final determination, not only of the number of chromosomes in a large group of species but also the details of their morphology, including the history of the SAT-chromosomes. The findings of Sato (1937) in *Aloinae* definitely show that the results of Taylor (1925) as regards the number of satellites were not final. Bruun (1932) did not find satellites in *Primula seclusa*, although they are present in the related species. But the development of nucleoli in that species suggests that the satellites may be very small. The presence of such small satellites, which can easily escape notice, has been demonstrated in *Haworthia*, *Trillium*, &c. In spite of these drawbacks important advance has been made in this direction.

De Mol (1926), from his root-tip study in *Hyacinthus*, found that the diploids have two, triploids three, and tetraploids four nucleoli. Thus the number of nucleoli, which probably corresponds to those of the satellites, gave a reliable indication of polyploidy in the genus. Later this assumption has been supported by a number of investigators, though there are exceptions also which require further interpretation.

Heitz (1931) has shown in diploids the presence of two satellites and two nucleoli in telophase. Navashin (1927) found four satellites, two large and two small, attached to the nucleolus in a tetraploid *Galtonia*. McClintock (1934) showed diploid and triploid *Zea Mays* with two and three nucleoli and satellites respectively. Geitler (1932) showed the presence of four satellites and four nucleoli in *Crepis capillaris*. Ramanujam (1937) found three

nucleoli, probably corresponding to three satellites, in an autotriploid rice. Bhatia (1938) has very definitely demonstrated the presence of four satellites and nucleoli in a tetraploid wheat, *Triticum dicoccum*, and six satellites and nucleoli in the hexaploid *Triticum vulgare*. The unequal size of the nucleoli and satellites suggested that they are both allopolyploids.

The results of Resende (1937) and Sato (1937), who find in diploids more than two satellites and nucleoli, may find an explanation in secondary polyploidy, as Gates (1938) has suggested for some of the *Lactuca* species studied by Babcock, Stebbins, and Jenkins (1937). Woods (1937) found six satellites and nucleoli in one strain of *Tulipa* with twenty-four chromosomes, and in others an even larger number of nucleoli. Sinoto (1938) found in *Paeonia* ( $2n = 10$ ) eight satellites and nucleoli. The above two examples require special attention, and perhaps extreme hybridisation may have played a role in bringing out such exceptional conditions.

However, the *Crocus* species examined here strongly support de Mol's hypothesis.

## 6. SUMMARY

1. Twelve species and two varieties of *Crocus* have been examined. For chromosome numbers see Table I, p. 231; the numbers found tallied with those of the previous investigators in all cases except in *Crocus susianus* ( $2n = 12$ ). Evidence from both somatic and meiotic studies shows that this species is a secondarily-balanced diploid.

2. The morphology of the somatic chromosomes has been studied and a great variation in the size of the chromosomes possessing median, sub-median, and sub-terminal primary constrictions was observed.

3. Satellites and nucleoli have been observed for the first time in nearly all the species examined. The number of satellites varies from 2 to 6 in different species. A great variation in their size was found. The maximum number of nucleoli in telophase corresponds in each species with that of the satellites, proving Heitz's hypothesis. The size of the nucleoli also varies with that of the satellites.

4. In the majority of the species organization of nucleoli was observed. In early telophase many small droplets appear first, probably derived from the matrix of all the chromosomes. They are later collected on the satellite thread by the activity not only of the organizer but presumably of the satellite also. Thus by the end of the telophase nucleoli corresponding to the number of the satellites or secondary constrictions are usually formed, but in many cases they fuse quite early.

5. A polyploid series has been established for *C. speciosus* var. *albus* ( $2n = 12$ ), possessing four satellites and four nucleoli in telophase, and *C. speciosus* ( $2n = 18$ ), possessing six satellites and six nucleoli in telophase. They are thus respectively tetraploid and hexaploid forms, the basic number being three, which corresponds to the lowest haploid number in the genus.



Somatic doubling in chromosome number was observed in *C. Olivieri* ( $2n = 6$ ) and *C. sativus* var. *Elwesii* ( $2n = 15$ ). In the latter, very clear sectorial chimaeras are present showing approximately a doubling in the size of the cells, nuclei and nucleoli.

6. Prochromosomes have been found in five species and in one variety with fairly long chromosomes.

7. Meiosis has been studied in (i) *C. susianus* ( $2n = 12$ ) where usually six bivalents are formed at the heterotypic division; but sometimes a ring of four chromosomes was observed and a probable explanation for the same has been given: (ii) *C. ochroleucus* ( $2n = 10$ ) where five bivalents are formed at the heterotypic division. Sometimes chromatid bridges due to the presence of inverted segments were observed both at anaphases I and II.

8. The relation of satellites and nucleoli to phylogeny is discussed.

## 7. ACKNOWLEDGEMENTS

I wish to acknowledge my deep indebtedness to Professor R. R. Gates, and express my grateful thanks for his unfailing interest, valuable guidance and kind criticism throughout the progress of this investigation. I am also grateful to Mr. C. S. Semmens for taking the photomicrograph.

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# Observations on the Anaerobic Respiration of Potato Tubers

BY

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With four Figures in the Text

THERE exists a considerable body of literature on the relationship between aerobic and anaerobic respiration. In particular, a number of workers have reported that, on transferring tissues from air to nitrogen, the output of carbon dioxide increased above the 'air line' value, remained at that higher level for varying lengths of time, eventually falling away and passing below the air line value. Such results have been reported by Gustafson (1930) for the tomato, G. R. Hill (1913) for grapes, cherries, and blackberries, Choudhury (1939) for the carrot, and Parija (1928) for the apple. The theory of oxidative anabolism which Blackman (1928) based on Parija's observations on the apple has given added interest to the examination of the carbon dioxide output of tissues immediately after transference from air to nitrogen.

Our observations on potato tubers gave the familiar form of curve, a temporary increase in carbon dioxide output on passing from air to nitrogen, subsequently falling away to an almost constant anaerobic value, about 50 per cent. of the aerobic value. Fig. 1 illustrates the form of curve obtained. The amount of carbon dioxide evolved in excess of the air line value was, however, small compared with the values obtained from the apple. During earlier experiments on the respiration of slices of washed tissue we observed that the tissue contained a considerable amount of chemically combined carbon dioxide and that the amount varied with the experimental conditions. The work recorded in this paper is concerned with the amount of this bound carbon dioxide, the factors which control the amount, the effect of external conditions, and the way in which changes in the content would be reflected in the output of carbon dioxide from the tuber. The results obtained have led to an explanation, other than that on a basis of 'oxidative anabolism', of the form of the carbon dioxide output curve from whole tubers in the early stages of anaerobic respiration.

## RESULTS

This section can be divided into two parts, the first concerning the whole tuber, i.e. the estimation of carbon dioxide output and the carbon dioxide content of the internal atmosphere of the tuber, the second concerning sliced tissue, i.e. the respiration of tissue slices and the determination of the factors controlling the amount of bound carbon dioxide.

The tubers used, mainly 'red' varieties, in following the carbon dioxide output curves, were kept at a working temperature, 29° C., in a thermostat in a constant current of air (4 litres per hour) for two days before readings

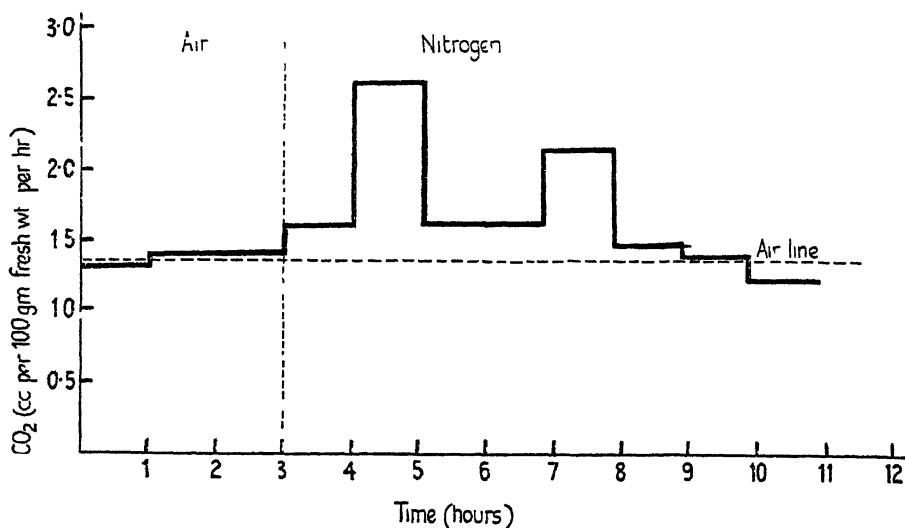


FIG. 1. Output of carbon dioxide from whole potato tubers in air and nitrogen showing the amount in excess of the air line after transference from air to nitrogen.

were taken. The rate of gas flow was maintained at a constant value, the rate being measured with the aid of a calibrated flow-meter. The air was freed from all traces of carbon dioxide and warmed to the temperature of the bath before entering the respiration chamber. The carbon dioxide in the output air was absorbed in N/20 baryta in Pettenkofer tubes. Aliquots of the solution were titrated against N/40 HCl, using thymol blue as an indicator, the end point being determined by the development of a neutral grey shade. The titrations were carried out in narrow boiling-tubes fitted with corks cut to allow the passage of the burette jet and of a mechanically operated glass stirring-rod.

The nitrogen used during the anaerobic experiments was freed from oxygen by passage through an alkaline solution of sodium hydrosulphite and subsequently washed by passage through strong soda and water. The solution of sodium hydrosulphite was made up as follows: 50 gm. sodium hydrosulphite

in 250 c.c. of water with the addition of 40 c.c. of a solution of sodium hydroxide (500 gm. NaOH in 700 c.c. water). This solution is the most efficient of oxygen absorbents, 1 gm. of sodium hydrosulphite absorbing 128 c.c. of oxygen. This solution was made up in quantities of 4 litres with two Winchester quart bottles as absorption flasks; such quantities last a very long time.

The transition from air to nitrogen was accomplished with the aid of a three-way tap which results in a minimum disturbance of the gas flow.

Fig. 1 shows one of a series of curves obtained using tubers of a 'red'

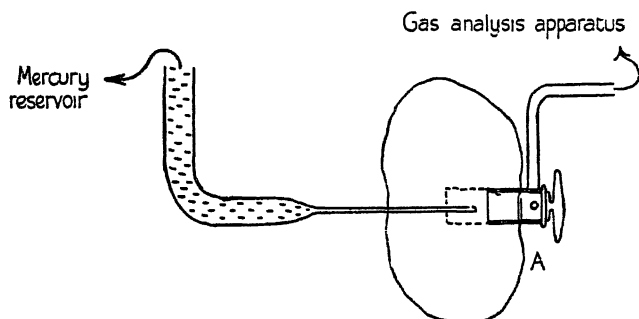


FIG. 2. Shows the insertion of the sampling chamber in the tuber and the method of transferring the gas sample from the tuber to the gas analysis apparatus.

variety during the autumn and winter. The curve was started on February 15, 1939. For this group of tubers the carbon dioxide in excess of the air line was 2.80 c.c. per 100 gm. fresh weight. The output from the 13th to the 28th hour after the commencement of the anaerobic conditions averaged 0.73 c.c. per 100 gm. fresh weight per hour, i.e. 54 per cent. of the original air line value, the values during the 31st and 32nd hours being 0.70 c.c. and 0.62 c.c. respectively. In general, the amount of carbon dioxide in excess of the air line varied with the variety of potato and the time of year, being greatest with 'red' varieties used during the autumn and winter, and least with early varieties used towards the end of April and beginning of May.

The determination of the concentration of carbon dioxide in the air spaces of the tuber was carried out by inserting a glass tube, about 1 cm. wide and 1.5 cm. long and closed at one end by a hollow stopper tap, into a pit cut into the tuber. The diameter of the pit was such that the glass tube fitted closely, a mixture of vaseline and lanoline being smeared on the surface of the tuber at the glass-tuber interface. The pit extended to the central line of the tuber. After eighteen hours the gas in the tube was displaced by mercury, as shown in Fig. 2. A hollow glass needle, made by drawing out a piece of stout walled glass tubing and attached by rubber tubing to a reservoir of mercury, was pushed through the tuber from the side opposite to the gas sampling chamber until it entered the chamber. This chamber was connected to the gas analysis



apparatus by a tube filled with mercury. On opening tap *A* and raising the reservoir the gas sample was transferred to the gas analysis apparatus. The use of a small positive pressure to transfer the gas removes any risk of dilution due to leaks. The glass needle used does not become blocked with tissue as it is forced through the tuber if its end is rounded and the aperture laterally placed. Using tubers weighing about 70 gm., the average concentration of carbon dioxide in the internal atmosphere was 11.6 per cent., at 29° C.

The total gaseous space in a tuber was determined by cutting cylinders from a tuber of known weight, infiltrating with absolute alcohol, and collecting the gas given off. By calculation from the weight of the tuber, the weight of the residue after the cylinders had been removed, and the volume of gas given off by the cylinders, the gaseous space per 100 gm. fresh weight was determined. The method is not one of great accuracy, as the gas collected is not of exactly the same constitution as that of the internal atmosphere as determined by the method previously described. This difference is due to the difference in solubility of the gases concerned in the aqueous phase of the tuber and in 45 per cent. alcohol, the usual concentration of the spirit at the end of the experiment. A number of results averaged at 6.5 c.c. per 100 gm. fresh weight.

The determination of bound carbon dioxide and the respiration rates of washed sliced tissue were carried out as described by us in an earlier paper (Boswell and Whiting, 1938), using the Warburg manometric technique. The slices used were 0.025 in. thick, were washed for at least two days and suspended in distilled water in the cups. The bound carbon dioxide was liberated by the addition to the tissue of 0.3 c.c., 10 per cent.  $\text{H}_2\text{SO}_4$ .

The first group of experiments were to determine the relationship between the amount of bound carbon dioxide in the tissue and the partial pressure of carbon dioxide in the atmosphere in contact with the tissue. Fig. 3 illustrates this relationship, and Table I gives a comparison between the bound carbon dioxide in washed slices and in freshly cut slices under the same partial pressure of carbon dioxide.

TABLE I

*Bound  $\text{CO}_2$  (c.c. per 100 gm. fresh wt.)*

Partial pressure of $\text{CO}_2$ (%).	Washed slices.	Fresh slices.
Air	0.8	0.7
	1.2	1.4
4.1	6.1	—
	6.3	—
5.0	6.9	4.8
	7.0	5.1
10.0	7.9	—
20.0	11.4	12.9
	11.8	—

It is of interest to note that the binding power of the washed and fresh slices was of the same order.

Table II contains examples of results obtained from a second group of experiments, which were designed to determine the effect of anaerobic conditions on the capacity of washed slices to bind carbon dioxide chemically.

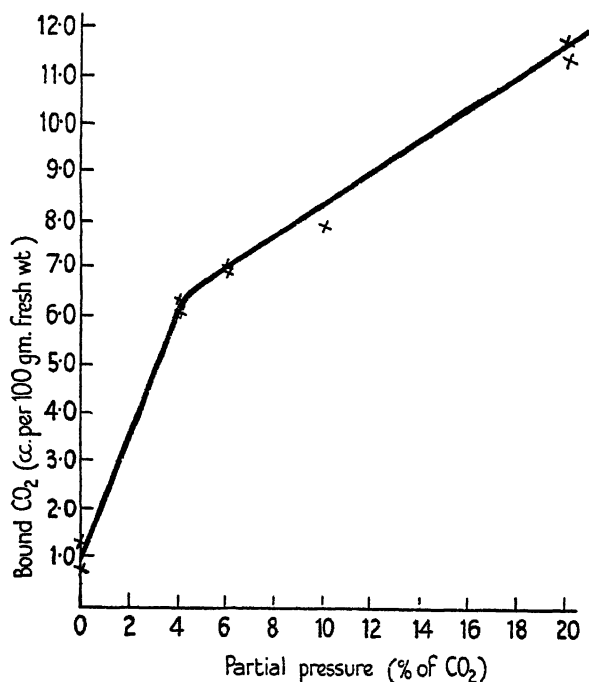


FIG. 3. Relationship between the partial pressure of carbon dioxide and the amount of the gas bound chemically.

Weighed groups of slices 0.025 in. thick were placed in the cups of Warburg manometers, the air being displaced with a gas of known constitution. After exposure to this atmosphere for one hour the bound carbon dioxide was determined by the method previously described. It had been previously found that the maximum effect of the anaerobic condition on the basicity of the tissue was obtained after that period, and further exposure did not result in any further decrease in the basicity. The differences between the aerobic values are due in part to differences between individual tubers and in part to the samples being taken at different times of the year.

In order to make comparisons between the basicities of tissue under aerobic and anaerobic conditions it is necessary to calculate the amounts of bound carbon dioxide under the same partial pressure of carbon dioxide. This can be done by means of the data in Table I.

TABLE II

*Bound CO<sub>2</sub> (c.c. per 100 gm. fresh wt.)*

Aerobic mixture: 10<sup>0</sup>/<sub>0</sub> O<sub>2</sub>, 4.12<sup>0</sup>/<sub>0</sub> CO<sub>2</sub> } in N<sub>2</sub>  
 Anaerobic mixture: 0.0<sup>0</sup>/<sub>0</sub> O<sub>2</sub>, 4.76<sup>0</sup>/<sub>0</sub> CO<sub>2</sub> }

Sample 1.		Sample 2.	
Aerobic.	Anaerobic.	Aerobic.	Anaerobic.
5.76	5.20	6.71	5.27
5.56	4.87	6.20	5.59
5.61	4.53	6.41	5.25
—	5.12	—	5.06
5.64	4.93	6.43	5.29

Aerobic mixture: 10<sup>0</sup>/<sub>0</sub> O<sub>2</sub>, 4.67<sup>0</sup>/<sub>0</sub> CO<sub>2</sub> } in N<sub>2</sub>  
 Anaerobic mixture: 0.0<sup>0</sup>/<sub>0</sub> O<sub>2</sub>, 6.10<sup>0</sup>/<sub>0</sub> CO<sub>2</sub> }

Sample 3.

Aerobic.	Anaerobic.
9.58	6.86
9.50	6.82
9.45	6.89
—	6.53
9.53	6.77

An increase in the partial pressure of carbon dioxide from 4 to 5 per cent. increases the bound carbon dioxide by 0.9 c.c.; while, between 5 and 10 per cent., each 1 per cent. increase in the partial pressure raises the bound carbon dioxide content by 0.2 c.c. The aerobic values in samples 1 and 2 are calculated as if under a partial pressure of 4.76 per cent. CO<sub>2</sub>, and in sample 3 the anaerobic value is reduced to the level of 4.67 per cent. CO<sub>2</sub>. Table III gives the adjusted values.

TABLE III

*Bound CO<sub>2</sub> (c.c. per 100 gm. fresh wt.)*

	Sample 1.	Sample 2.	Sample 3.
Aerobic	6.22	7.01	9.53
Anaerobic	4.93	5.29	6.48

The decrease in the capacity to bind carbon dioxide chemically after exposure to anaerobic conditions is clearly marked and would result in the liberation of 1.29 c.c., 1.72 c.c., and 3.05 c.c. per 100 gm. fresh weight from the three samples during the first hour of anaerobic respiration.

The wide differences which exist between different tubers, even of similar size and history, in respect of their capacity to bind carbon dioxide under a constant partial pressure of the gas, precludes any chance of confirming these

results using whole tubers taken from a population before and after exposure to nitrogen.

It is possibly of interest to include some results obtained using the unicellular alga, *Chlorella*, with the same technique. The cultures used were mature with a high cell concentration, and were kept at 25° C. for two hours in contact with air and with nitrogen. The bound carbon dioxide was determined in the usual manner, and the results are given in Table IV.

TABLE IV

*Bound CO<sub>2</sub> (c.c. per 5 c.c. of algal suspension)*

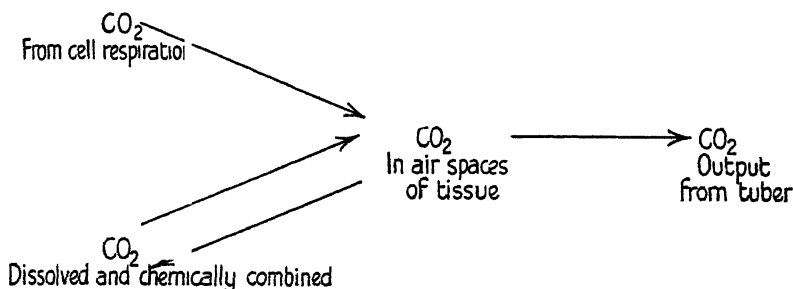
<i>Air.</i>	<i>Nitrogen.</i>
0.0589	0.0306
0.0618	0.0320
0.0595	0.0336
	0.0343

The decrease in the basicity of the tissue is almost 45 per cent.

The results recorded give some picture of the carbon dioxide relationships within the potato tuber, and in the following discussion an attempt is made to link them together to explain the form of the anaerobic carbon dioxide curves in the early stages.

#### DISCUSSION

The diagram below expresses the relationship which it is clear must exist within any bulky tissue between the carbon dioxide output and the carbon dioxide produced by cell respiration.



The output of carbon dioxide from the tuber is dependent upon two factors: (1) the permeability of the tissue to the gas, of which little is known; (2) the partial pressure of carbon dioxide in the air spaces. Under constant external conditions over short periods of time this partial pressure is determined by the rate of carbon dioxide production by the cells. The carbon dioxide in the air spaces is in equilibrium with the carbon dioxide bound in the tissue, and is one of the factors determining the amount bound. This amount is also controlled by the basicity of the tissue. The basicity is due to the presence

within the tissue of salts which form bicarbonates and amino acids and proteins with which carbon dioxide forms complex units. The amount of bound carbon dioxide is considerable. Table I shows that in tubers with an internal atmosphere containing 11.6 per cent. carbon dioxide the amount of bound carbon dioxide will be of the order of 8.0 c.c. in 100 gm. fresh weight. The values for the carbon dioxide content of the internal atmosphere, given by Magness (1920), are much in excess of our values, being at 11° C., 19.6 per cent. CO<sub>2</sub>, and at 22° C., 34.4 per cent. CO<sub>2</sub>. This may be due in part to the differences in technique. He drew samples of gas from the intercellular spaces by applying a negative pressure, and it is probable that under these circumstances much of the bound carbon dioxide is decomposed and appears in the gas drawn from the air spaces.

It is clear that with such a large quantity of carbon dioxide bound in the tissue, and partially dependent upon the partial pressure of carbon dioxide within the air spaces, that in any change in the external conditions which results in a change in the rate of production of carbon dioxide—and hence of concentration in the intercellular spaces—account must be taken of changes which occur in the content of bound carbon dioxide and also of the influence of the external conditions on the basicity of the tissue through changes in the nature of the end products of respiration.

Such a change in the external conditions is the transference from aerobic to anaerobic conditions. It is therefore valuable to consider the form of the carbon dioxide output curve under anaerobic conditions, immediately after the change from the aerobic, in the light of the changes in the basicity of washed slices exposed to anaerobic conditions.

As expressed diagrammatically on p. 263, provided the permeability of the tissue to the gas remains constant, changes in the rate of carbon dioxide output reflect changes in the partial pressure of the gas in the intercellular spaces. Any increase in the rate of carbon dioxide output can only be brought about by an increase in the partial pressure of the gas in the air spaces. In order to determine the cause of the rise in the rate of output under anaerobic conditions it is necessary to determine the source of the increased carbon dioxide content of the intercellular air spaces. There are two possibilities—either the rate of carbon dioxide output from the cells is increased—this is in part the basis of Blackman's theory of 'oxidative anabolism'—or some bound carbon dioxide must be liberated due to a decrease in basicity.

Leaving on one side the first alternative it is proposed to consider how far the form of the anaerobic respiration curve can be explained as the resultant of the reduced carbon dioxide output of the cells due to the anaerobic conditions, and of production of carbon dioxide resulting from certain changes in the basicity of the tissue which we have observed under anaerobic conditions. The rate at which the curve would fall away due to the reduced carbon dioxide output of the cells is to a large extent offset during the first few hours by the liberation of bound carbon dioxide due to the falling internal concentra-

tion of the gas as the result of reduced output from the cells. Furthermore there is the gaseous carbon dioxide in the intercellular spaces to act as a reservoir.

While these sources of carbon dioxide act as a reserve, making the transition from aerobic to anaerobic rates of output gradual, neither source can increase the concentration of carbon dioxide in the air spaces above the value existing during aerobic respiration. It is therefore necessary to consider the changes, if any, which occur in the basicity of the tissue as the result of anaerobic respiration, and to decide if these changes are of such a nature and of sufficient magnitude to account for the output of carbon dioxide in excess of the air line value during the early stages of anaerobic respiration.

In considering this relationship it is first necessary to show that under anaerobic conditions the basicity of the tissue of a potato tuber is reduced, and, secondly that the amount of carbon dioxide which would be liberated through the reduction in basicity is of the same order as the carbon dioxide evolved in excess of the air line value. As a result of the balancing effect on the carbon dioxide output rate of the gas in the air spaces and of that liberated from the combination with certain absorbents in the tissue due to a falling internal partial pressure, the air line value may, during the early hours of anaerobic respiration, be regarded as the basic rate on which the excess output is superimposed. The values recorded in Table II make it clear that as the result of exposure to anaerobic conditions the basicity of the tissue is reduced. From the values given in Table III it was calculated that the amounts of bound carbon dioxide which would be liberated through the reduction in basicity of the tissue are 1.29, 1.72, and 3.05 c.c. per 100 gm. fresh weight. These values were calculated from experiments made with partial pressure in the neighbourhood of 5 per cent. carbon dioxide. If these values had been determined under a partial pressure of  $\text{CO}_2$  of 12 per cent., that of the internal atmosphere of the tuber, they would, calculating on the basis of the values in Table I, have been larger by about 30 per cent., the amounts liberated being 1.68, 2.24, and 3.97 c.c. per 100 gm. fresh weight. To compare these values obtained from washed, sliced tissue with values of the output of carbon dioxide in excess of the air line from tubers under anaerobic conditions involves the comparison of results obtained from whole tubers with those of sliced tissue. In support of the soundness of this comparison, it may be seen in Table I that washed slices and fresh-cut slices have basicities of the same order, and this in spite of wide differences in their rates of respiration. The basicity of the tissue does not, therefore, appear to be affected by any wound reaction or by the rate of respiration of the tissue. The basicity of the tissue appears to be due to substances within the cells which are unaffected by the washing of the slices in running water, the ions which are lost from the cells during this period apparently have little part in controlling it. The higher rate of respiration of slices as compared with whole tubers is probably due to increased availability of oxygen, and this does not apparently affect the

basicity of the tissue. On this evidence we consider that it is reasonable to compare the results which have been obtained with washed slices with those for whole tubers, in these matters of changes in the basicity of tissue slices under anaerobic conditions, and the form of the whole tuber carbon dioxide output curve under similar conditions.

With 'red' varieties of potatoes, the following values have been obtained of the amounts of carbon dioxide given off under anaerobic conditions in excess of the air line, 1.50, 1.15, 1.31, and 2.80 c.c. per 100 gm. fresh weight. With early varieties used after the end of April 1939, the excess values were much smaller, i.e. 0.24 and 0.57 c.c. per 100 gm. fresh weight. It is clear, therefore, that the amount of carbon dioxide given off in excess of the air line values during the early hours of the anaerobic respiration of potato tubers, is of the same order as the amount of carbon dioxide which is evolved by washed slices due to a decrease in the basicity of the tissue under similar conditions. Table V brings the values together and makes this point clear.

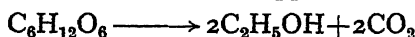
TABLE V

*CO<sub>2</sub> (c.c. per 100 gm. fresh wt.)*

CO<sub>2</sub> in excess of air line—whole tubers—1.50, 1.15, 1.31, 2.80  
CO<sub>2</sub> through decrease in basicity of slices—1.68, 2.24, 3.97

The decrease in the basicity of the tissue under anaerobic conditions can have only one source, the production of acid by the tissue. Excretion of positive ions into the distilled water in which the slices are suspended in the cups would not affect the result as the manometer cup and its contents are a closed system in respect of each determination of bound carbon dioxide.

It is already known from the observations of Boysen Jensen (1933) that the potato produces very little alcohol under anaerobic conditions. This is in keeping with our observation that under such conditions they produce acid, and the extension of this observation to *Chlorella* suggests that acid production under anaerobic conditions may be widespread. The anaerobic respiration of the potato tuber cannot, therefore, approximate to



and therefore any conclusions based on the ratio of anaerobic to aerobic carbon dioxide as to the existence of an 'oxidative anabolism' under aerobic conditions are valueless until more is known of the end products of anaerobic respiration in this tissue.

Finally, the differences in form between the anaerobic carbon dioxide output curves of washed slices (Fig. 4) and of whole tubers (Fig. 1), in particular in respect of the absence of any initial acceleration in the former, may be considered in the light of our observations already recorded. The absence of any initial acceleration on transference of sliced tissue from aerobic to anaerobic conditions is to be explained by the absence of any appreciable

amount of bound carbon dioxide in the tissue. The low level of bound carbon dioxide is due to the small partial pressure of the gas in the nitrogen with which the slices are in contact and the absence of any intercellular spaces containing

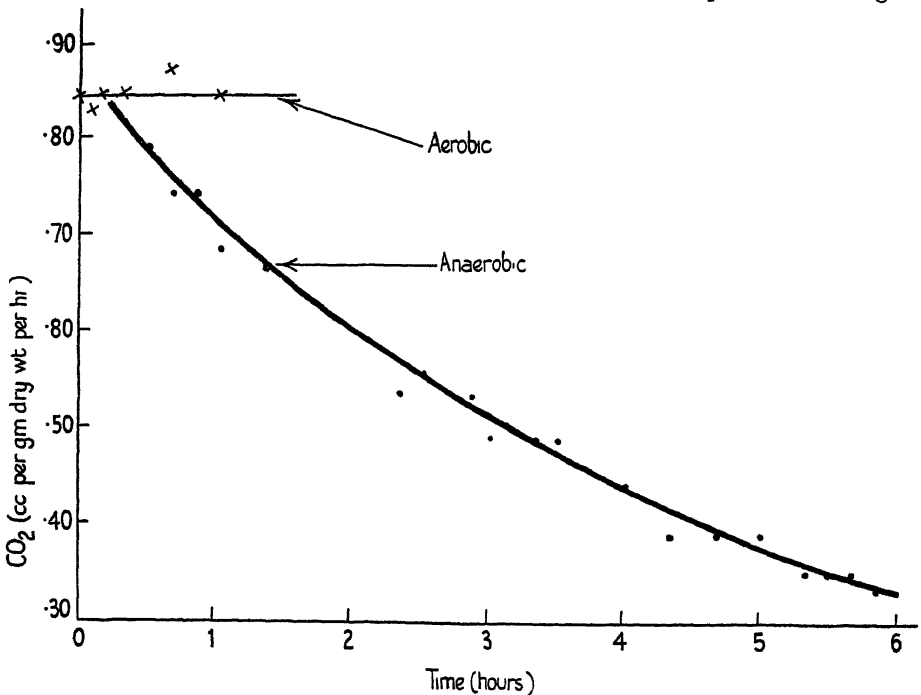


FIG. 4. Carbon dioxide output from washed slices, 0.025 in. thick, under aerobic and anaerobic conditions.

a high content of carbon dioxide as in the whole tuber. The absence of any large quantity of bound carbon dioxide would also explain the rapidity of the fall in carbon dioxide output during the first few hours of exposure to nitrogen.

### *Conclusion.*

As far as the potato tuber is concerned, the results recorded in this paper suggest that neither the form of the carbon dioxide output curve during the hours immediately after transference of the tubers from air to nitrogen, nor the ratio of anaerobic to aerobic carbon dioxide output is evidence of the existence of oxidative anabolism during aerobic respiration. The form of the carbon dioxide output curve for whole tubers under anaerobic conditions is considered as the resultant of the output of the gas from the cells and that liberated from chemical combination with the tissue due to a decrease in the basicity of the tissue under anaerobic conditions. On this interpretation the rate of carbon dioxide output from the cells is always lower under anaerobic conditions than under aerobic.



We desire to emphasize that this interpretation is only regarded as valid so far as the potato tuber is concerned, and we are not at present prepared to suggest that this is the universal explanation of the respiratory curves which led to the development of the theory of oxidative anabolism. It must, however, be emphasized that in any comparisons between carbon dioxide outputs from bulky tissues under aerobic and anaerobic conditions, account must be taken of changes in the bound carbon dioxide content and in the carbon dioxide content of the internal atmosphere when these may be of such an amount as to affect determination of the respiratory activity of the tissue.

### SUMMARY

The form of the carbon dioxide output curves from potato tubers immediately after transference from air to nitrogen is examined in the light of certain observations on the content of bound carbon dioxide of washed slices which have been under anaerobic conditions. The anaerobic conditions result in a decrease in the basicity of the tissue and the resultant liberation of amounts of carbon dioxide which are of the same order as those evolved in excess of the air line by potato tubers under similar conditions. In the case of potato tubers there is thus no need to assume the existence of oxidative anabolism.

We desire to thank Professor W. H. Pearsall for the interest which he has shown during the course of the work and the many ways in which he has facilitated it.

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# Studies in Tropical Fruits

## IX. The Respiration of Bananas during Ripening at Tropical Temperatures

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With twenty-three Figures in the Text

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## I. INTRODUCTION

IN these studies it is held that in the investigation of the storage behaviour of tropical fruits it is desirable to include biochemical and physiological studies of the changes undergone during development on the tree. With regard to the Gros Michel banana the former has already been undertaken (Barnell,

1940a); preliminary observations which have been made on respiration indicate that the problem is one of considerable complexity—a conclusion which is perhaps not unexpected when the unusual features of the formation and development of the banana bunch are considered (Wardlaw, Leonard, and Barnell, 1939).

The transport of bananas in cool storage may be regarded as being essentially a prolongation of the pre-climacteric, unripe phase in incompletely developed fruit. To understand the nature and extent of the changes which have taken place as a result of subjecting fruit to refrigeration some standard of comparison is necessary. Accordingly the initial studies described here have been carried out at tropical temperatures using fruit from bunches which were approaching their full development on the plant, the view being held that the behaviour of such fruit affords the nearest approximation to what may be termed 'normal ripening' in a state of nature. (Since the Gros Michel banana does not ripen in a state of nature while attached to the tree and in the absence of disease or injurious agents, the term 'normal ripening' must be accepted with qualification.) Further, it has been ascertained by physiological measurements that the ripening of such fruit at high temperatures is marked by clearly defined phases. In such a study favourable conditions are also afforded for observing the progress of ripening in terms of regional and organographic changes.

As a result of work carried out chiefly on the papaw—a large, many-seeded fruit with a well-developed central cavity—the writers have indicated that respiration should be investigated on a more comprehensive basis than that of carbon dioxide liberation alone (Wardlaw and Leonard, 1936*a*, 1939). In the present paper the observations, by contrast, have been made on a comparatively small, solid fruit, data being submitted on some interrelated aspects of respiration observed in the Gros Michel banana, harvested approximately at the commercial grades described as '¾-full' and 'heavy ¾-full',<sup>1</sup> and maintained during ripening at 85° F., using the methods and materials described in detail in previous papers (Wardlaw and Leonard, 1939; Wardlaw, Leonard, and Barnell, 1939). Respiration rates as determined by the liberation of CO<sub>2</sub> at the surface have been measured for individual 'fingers' only.

## II. RESPIRATION DURING RIPENING AT 85° F. (29.4° C.)

### (a) *Respiration rate of 'heavy ¾-full' fruits at 85° F. and 100 per cent. relative humidity.*

In these studies the correlated data have assumed a considerable complexity. By way of introduction, therefore, observations are submitted on the curve obtained when the rate of respiration was measured in a 'heavy ¾-full' fruit maintained throughout at 85° F. and 100 per cent. R.H. This type of curve affords a general expression of the respiration rate during ripening

<sup>1</sup> For explanation of terms see Wardlaw, Leonard, and Barnell (1939).

of a freshly harvested fruit. The respiration chamber and method used with the several precautions necessary have already been described (Wardlaw and Leonard, 1939).

*Citation of data (Figs 1 and 2)*

Grade of fruit . . . . .	'heavy $\frac{3}{4}$ -full'
Weight of fruit at commencement . . . . .	148.8 gm.
" " conclusion of experiment (i.e. after 306 hours) . . . . .	138.6 gm.
Mean temperature . . . . .	(29.4° C.) 85° F.
Relative humidity . . . . .	100%
	mg./kg./hr.
Rate of respiration during pre-climacteric phase . . . . .	45
" " at climacteric peak . . . . .	273
" " during post-climacteric phase . . . . .	162
" " " rise . . . . .	195
" " during final senescent phase . . . . .	86

In Fig. 1 it will be seen that prior to the climacteric, respiration proceeded at an approximately steady rate, actually slightly decreasing. During this phase the fruit retained the palish green colour which had been noted at reaping. Before any critical colour change in the skin occurred the climacteric phase was initiated, being denoted in the respiration curve by a very rapid rise. As the climacteric peak value was approached the skin, which was still firm and turgid, showed the first indication of colour change to pale yellowish-green. The descent of the curve from the peak value was rapid, though less so than in the upward direction, finally reaching a fresh level, at which stage the skin had become pale yellow. The pale yellow condition was rapidly followed by ripening changes so that the whole fruit became ripe, soft, and limp, giving what has been recognized and described as the 'boiled' condition, a feature of banana ripening at high temperatures and humidities. It was during the descent from the climacteric peak that the 'sprung' condition, which varies slightly from fruit to fruit, occurred. (The 'sprung' condition consists in a softening of the hitherto turgid pulp and skin sufficient to permit of a perceptible response to pressure.) These ripening changes are in close agreement with those observed by Gane (1936 *a* and *b*) on fruit which had previously been transported in cold storage. From the 'sprung' condition onwards, according to the grade of fruit selected and the conditions maintained in the respiration chamber, some variation may be encountered. In the individual record under discussion the post-climacteric phase was marked by a slight rise at which point the first signs of brown mottling of the skin—a well-marked phase in the onset of senescence—were observed. Thereafter with the onset of final senescence, which was accompanied by a progressive browning of the skin and semi-liquefaction of the pulp, the respiration rate fell to a comparatively low value. It is to be noted that once the brown mottled stage has been reached fruits may be subject to fungal spotting and the growth of superficial moulds generally. Nevertheless, in experiments comparable with that illustrated in Fig. 1 and others which have been

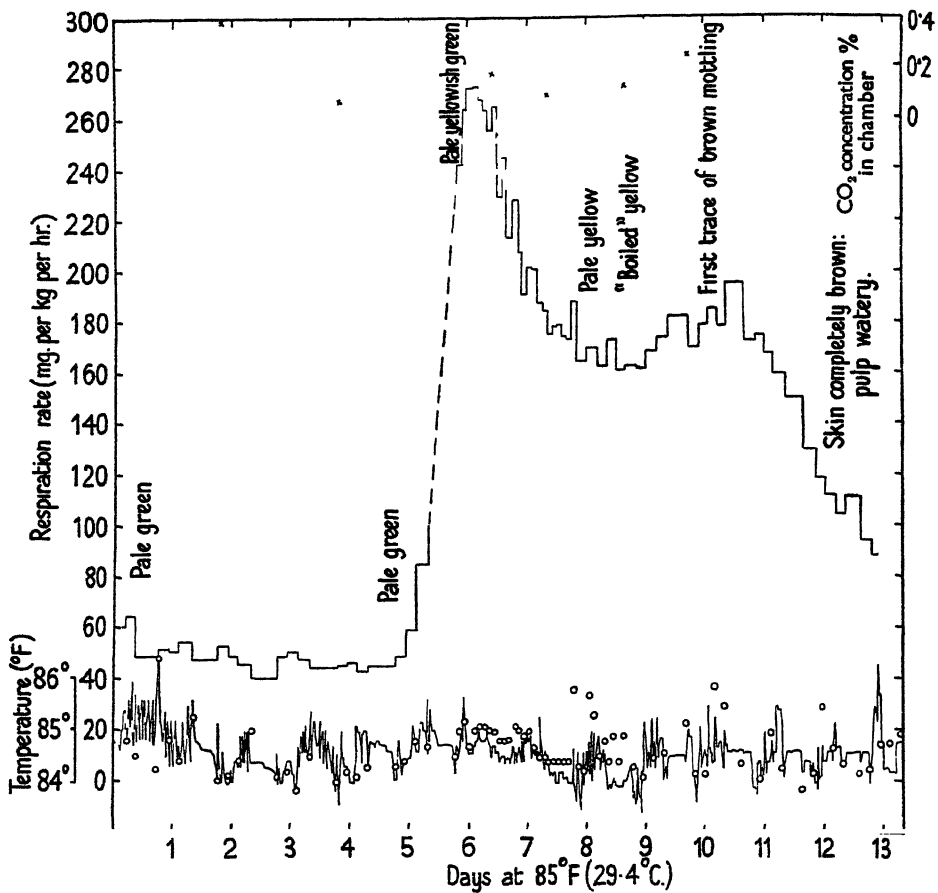


FIG. 1. Rate of respiration of a 'heavy 1/2-full' banana at 85° F. (29.4° C.) and 100 per cent. relative humidity. Thermograph and point records of temperature, and concentrations of CO<sub>2</sub> within the respiration chamber are also indicated.

undertaken in the course of these studies, such fungal activity has not been productive of any marked rise in respiration rate.

(b) *Respiration rates and internal concentrations of carbon dioxide and oxygen.*

In Fig. 2 the respiration rate values shown in Fig. 1 are reproduced as a smoothed curve and also, in conjunction, curves for the internal concentrations of carbon dioxide and oxygen for the same fruit, as obtained by the methods described in a previous paper (Wardlaw and Leonard, 1939).

During the pre-climacteric phase, the rise to the climacteric peak, and the initial region of the descent from the peak it will be observed that there is a

close correlation between the respiration-rate curve and that of internal carbon dioxide concentration.

Coincident with the beginning of the fall from the peak value, the curve for internal carbon dioxide concentration falls extremely rapidly. This in

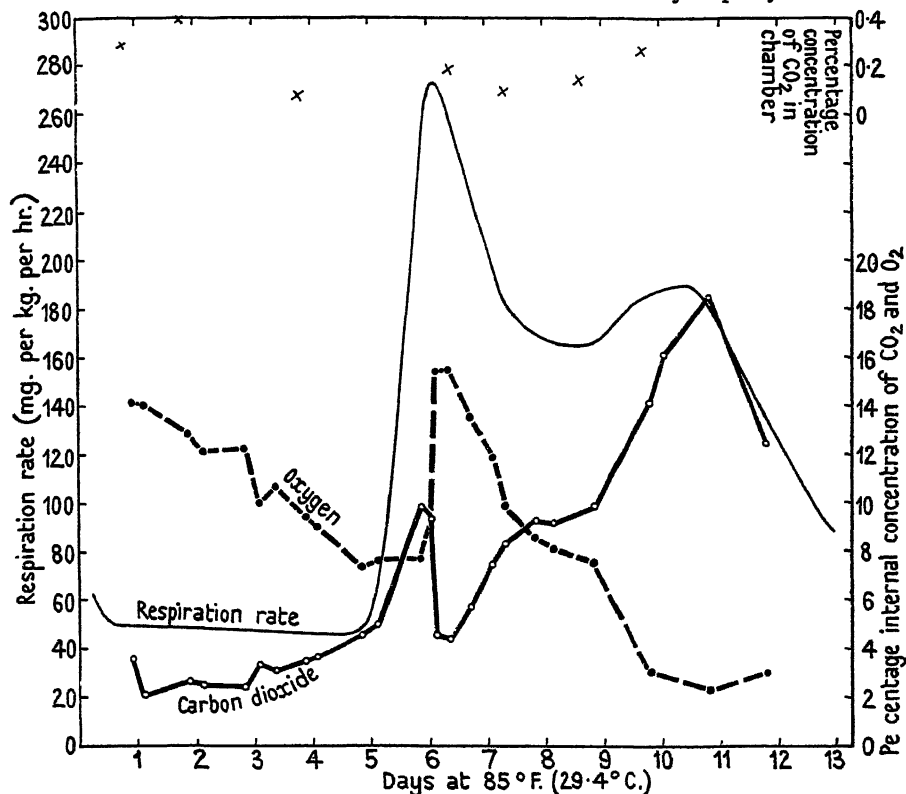


FIG. 2. Rate of respiration and internal concentrations of CO<sub>2</sub> and O<sub>2</sub> for the fruit of Fig. 1. Concentrations of CO<sub>2</sub> within the respiration chamber at different times are also indicated. The coincidence in time between the climacteric peak value in the respiration rate and in the internal concentration of CO<sub>2</sub> is to be noted.

turn is followed by a rise to a fresh high value which is maintained for approximately one day, under the conditions of this experiment, during which a correlation with the respiration-rate curve may again be observed. As from the stage in ripening which has been described and annotated as the 'boiled yellow' stage, a further rise in internal carbon dioxide concentration takes place. This is accompanied by a slight rise in the respiration-rate curve, but the proportionality is not maintained. In the final phase, the decline in the respiration-rate curve is accompanied by a decline in the internal concentration of carbon dioxide, but it will be shown later that this observation, whilst recorded in this particular instance, is a variable feature.

Turning to the internal oxygen concentration, it will be noted that prior

to the climacteric peak the curve descends to a low value, but quickly shows a marked recovery to a new high value; thereafter there is a fairly steady progressive fall in concentration until finally a low value (2–3 per cent.) is reached.

Particular features illustrated in Fig. 2 are (i) the low internal oxygen concentration coincident with the onset of the climacteric, its recovery and final decline, and (ii) the general correlation between internal carbon dioxide concentration and respiration rate until the later stages of senescence. Viewed as a whole, the important point to be noted is that whereas the pre-climacteric phase is marked by a low respiration rate, low internal carbon dioxide concentration, and comparatively high oxygen concentration, the post-climacteric phase is characterized by high respiration rate, high internal carbon dioxide concentration, and low oxygen concentration.

### III. THE RELATION OF TRANSPIRATION TO RESPIRATION

#### (a) *Discussion of factors involved.*

In respiration experiments with a 'draw-through' system the several variable factors within the respiration chamber are (i) temperature, (ii) gas concentration, (iii) relative humidity, and (iv) rate of change of air (Wardlaw and Leonard, 1939).

A respiring fruit, whether enclosed in a container or not, is not necessarily at the temperature of the surrounding air, nor are its different tissues at the same temperature. It has already been shown that although the temperature of the air surrounding the respiration chamber is approximately constant as is also the temperature of the air delivered and its rate of 'draw-through', nevertheless, because of changes in the metabolic activity of the fruit, the temperature of the air inside the chamber will be affected, and this in turn will react on the rate of metabolism. Fig. 3 shows the pulp temperatures during ripening in three comparable fruits (I) when the fruit was exposed on the bench to the atmosphere in the storage room, (II) when the fruit was in a respiration chamber supplied with air of a low relative humidity, and (III) when the fruit was in a respiration chamber supplied with air of a high relative humidity; the rate of air-supply in (II) and (III) was the same, the initial temperature of the air being that of the storage room as in (I); in each instance the temperature of the air surrounding the fruit is indicated.

Fig. 4 shows the differences between respiration chamber air temperature and pulp temperatures for fruits supplied with air of low and high relative humidities. Except during the climacteric phases, which approximately coincide in time, the flesh temperature was consistently higher in the fruit maintained at a high relative humidity.

Since transpiration tends to lower the temperature of the fruit, any modification of its rate, either by internal or external factors, will produce alterations in the fruit temperature and will therefore modify the rate of metabolism.

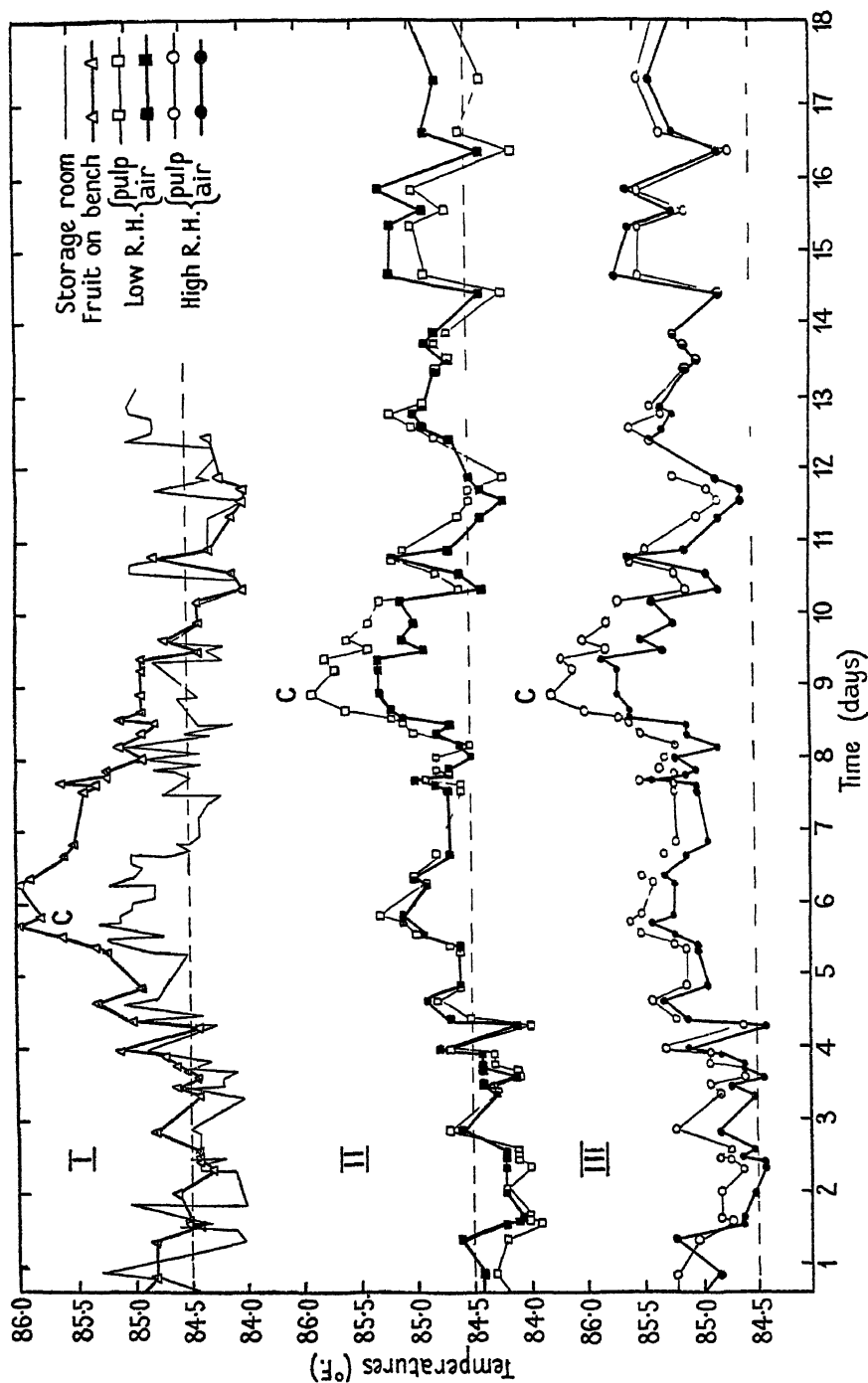


FIG. 3. Record of air and pulp temperatures during the ripening of '1-full' fruits at 85° F. and at different relative humidities. (see p. 274) C, climacteric peak.



Transpiration rate may be increased either by increasing the rate of air movement round a fruit (at relatively low rates of air movement) or by lowering the relative humidity of the air delivered.

A high rate of transpiration may not only affect metabolic processes by its effect on tissue temperature, but may also by its desiccating effect produce modifications in the tissues, whereby a restricting action on the movement of gases results; this in turn may be productive of additional changes in the metabolic trend. The high degree of mutual interaction of the several factors involved may thus be expected to produce important changes both in the ripening process and in the condition of the mature or senescent fruit.

Whilst considerable attention has been paid to the temperature factor during storage and to the gaseous composition of the atmosphere, the effect of relative humidity and air movement on transpiration (which, by the above showing, should be regarded as an integral part of metabolism) has been less exhaustively studied. Preliminary observations on transpiration during ripening which have been undertaken at this research station are in course of publication (Leonard, 1940).

*(b) Respiration rates at different relative humidities.*

As a preliminary investigation of the complex of factors considered above, the following experiment was carried out with the result shown in Fig. 5. Four comparable 'fingers' from the same 'hand' were placed in respiration chambers and provided with internal gas-sampling tubes. Air was supplied at relative humidities of approximately 65 per cent. and 95 per cent., using the methods already described (Wardlaw and Leonard, 1939), these humidities, however, being modified within the respiration chamber by the fruit's transpiration so that relative humidities of approximately 70 per cent. and 100 per cent. obtained. The rate of draw-through and the temperature of the surrounding atmosphere were kept constant. The respiration record at high humidity is that of Figs 1 and 2, at low humidity that of Fig. 7. Fig. 6 shows the respiration rate and humidity record for the duplicate finger to that of Fig. 7.

The following data for Figs. 6 and 7 are cited:

	Fig. 6.	Fig. 7.
Grade of fruit	'heavy $\frac{3}{4}$ -full'	'heavy $\frac{3}{4}$ -full'
Weight of fruit at beginning of experiment.	155.9 gm.	152.2 gm.
" " end of experiment.	117.1 gm.	102.8 gm.
Mean temperature	after 358 hours (29.4° C.) 85° F.	after 407 hours (29.4° C.) 85° F.
" relative humidity	70%	70%
	mg./kg./hr.	mg./kg./hr.
Rate of respiration during pre-climacteric phase	50	50
" " at climacteric peak	285	250
" " during post-climacteric phase	130	120
" " during final senescent phase	75	70

With the comment that the time of onset of the climacteric and the peak

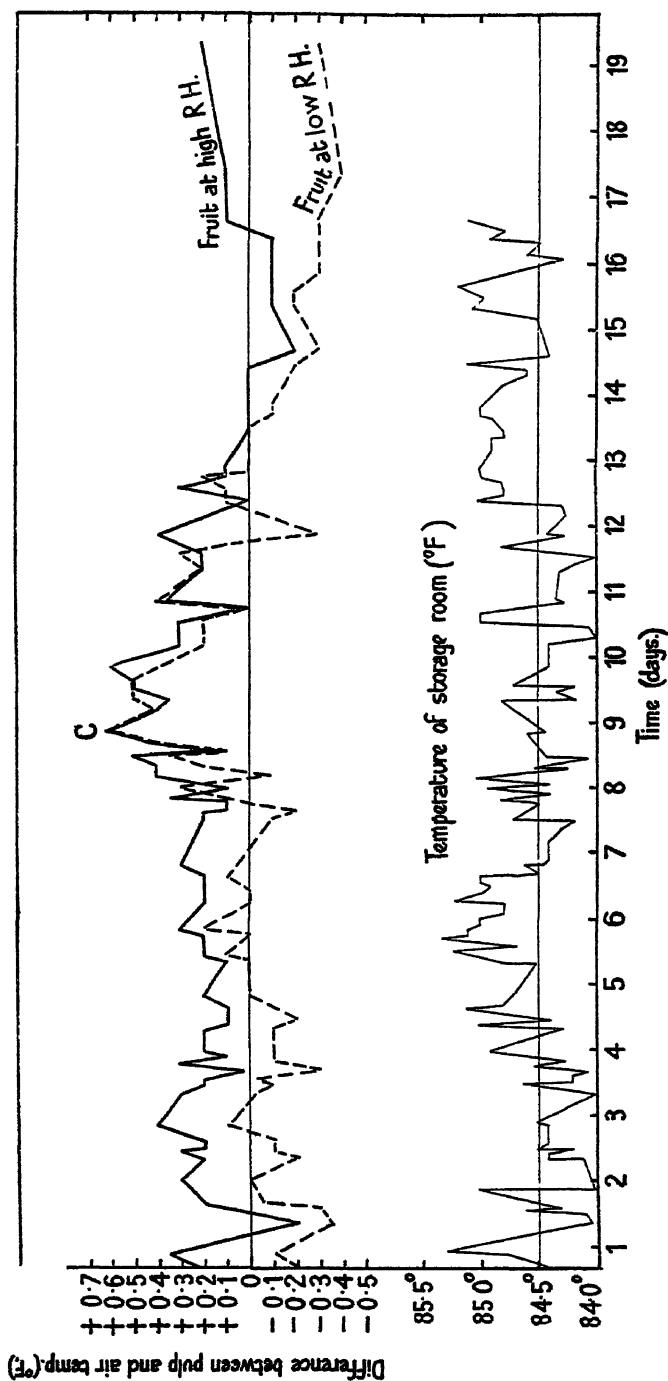


FIG. 4. Differences between pulp and air temperatures during the ripening of two '3-full' fruits held at the same room temperature but at high and low relative humidities. c, climacteric peak.

value of the respiration rate in fruits under identical conditions are variable, as indicated by other experiments, the principal points to be noted in this experiment, Fig. 5, are as follows. The pre-climacteric respiration rates are almost identical, while the rise to the climacteric peak value yields curves of essentially the same pitch. At the lower humidity the respiration rate curve shows an almost continuous decrease from the peak, whereas at the higher

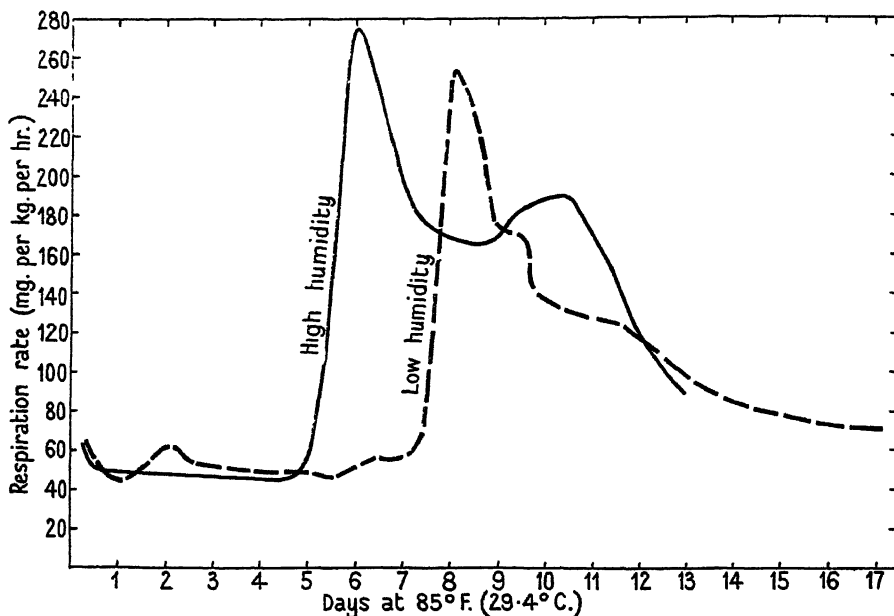


FIG. 5. Respiration rates during the ripening of 'heavy  $\frac{3}{4}$ -full' fruits at 85° F. and at humidities of 100 per cent. R.H. (continuous line), and of approximately 70 per cent. R.H. (broken line).

humidity an approximately constant post-climacteric level was maintained for three days. In the fruit at the lower humidity six days elapsed from the climacteric peak to the first appearance of anthracnose spots (Fig. 7), whereas at the higher humidity anthracnose appeared after four and a half days. The fruit held at low humidity was considerably desiccated at the end of the experiment. The lower respiration rate at all stages from the onset of the climacteric onwards at low humidity also indicates an important relation between desiccating factors and increased resistance to the movement of gases. The data illustrated in Fig. 6 confirm these general observations.

(c) *Effect of transpiration on internal gas concentrations.*

The internal gas concentrations for a fruit held at high relative humidity have already been discussed, Fig. 2. Those for fruit held at a lower relative humidity show considerable differences as illustrated in Fig. 7.

Tissue resistance to the movement of gases in fruit subjected to consider-

able desiccation is seen to be important. The internal carbon dioxide concentration in the pre-climacteric stage shows a slightly rising trend which is accompanied by a continuously and fairly rapidly descending curve of internal oxygen concentration. But whereas the curve of internal concentration of

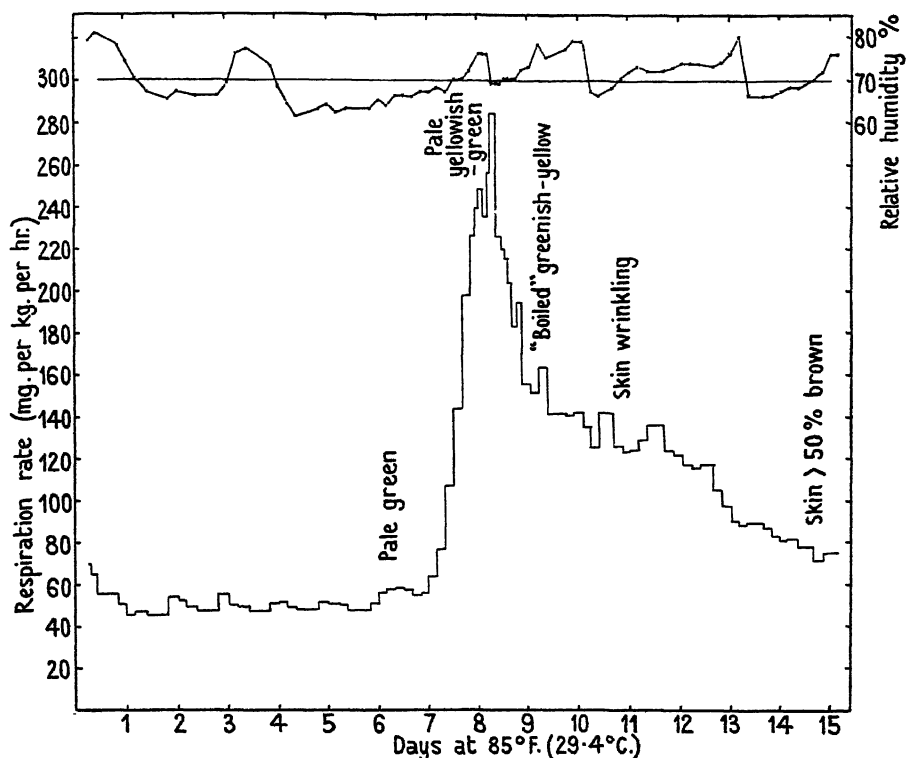


FIG. 6. Rate of respiration of a heavy '3/4-full' fruit at 85° F. (29.4° C.) and approximately 70 per cent. R.H. The record of relative humidity within the respiration chamber is also shown.

carbon dioxide showed a sharp rise and fall at the climacteric in fruits in a saturated atmosphere, Fig. 2, only a slight post-climacteric fall was observed in fruits at low humidity. There was likewise no evidence of the oxygen recovery which is such a marked feature of internal gas concentration records for fruits at higher humidities. In Fig. 8 the internal concentrations of carbon dioxide and oxygen in the fruits of Figs. 2 and 7 at high and low humidities respectively are shown. Both at the climacteric peak and subsequently, it will be noted that although the internal carbon dioxide concentrations in the fruit at low humidity were higher than those in the fruit at high humidity, the rates of respiration were considerably lower, again indicating changes in tissue resistance resulting directly from a greater degree of desiccation. Under conditions of low humidity the curve of internal carbon dioxide concentration shows very markedly the change over from a low to a

high internal concentration. The importance of extending these investigations to analyses of changes in metabolites during ripening, particularly to the fate of water in all its relationships, is strongly indicated. The relation between diminished water content and gas solution phenomena (Leonard, 1939) in a biological system is not sufficiently known, but together with the other factors enumerated may be far-reaching in its effects on the metabolic changes.

*(d) Effect of transpiration on pneumatic pressures within fruits.*

The effect of maintaining comparable fruits at high and low relative humidities has also been investigated in relation to changes in pneumatic pressure during ripening. This is dealt with in section V.

#### IV. INTERNAL CONCENTRATIONS OF CARBON DIOXIDE AND OXYGEN DURING RIPENING

*(a) General observations.*

The changing internal concentrations of carbon dioxide and oxygen in bananas during ripening in a storage room maintained at approximately 85° F. and 85 per cent. relative humidity were further investigated in a number of experiments in which fingers from the upper or lower row of the same hand were cut off, supplied with gas-sampling tubes, or attached to manometers containing Brodie's solution.

In Fig. 9, which shows the trends of internal concentrations of CO<sub>2</sub>, O<sub>2</sub>, and CO<sub>2</sub> *plus* O<sub>2</sub> during the ripening of a  $\frac{3}{4}$ -full fruit, the pre-climacteric phase is marked by a slow rise and by a slow decline in the concentrations of CO<sub>2</sub> and O<sub>2</sub> respectively. With the initiation of ripening on the eighth day there is a sharp decline in the O<sub>2</sub> concentration and a rise in the CO<sub>2</sub> concentration. Within twenty-four hours a well-defined recovery in oxygen concentration has taken place, the CO<sub>2</sub> curve meanwhile descending from the peak to a lower value. During the post-climacteric phase high O<sub>2</sub> values are maintained for one day, after which a well-marked decline takes place. This is accompanied by a second rise in CO<sub>2</sub> concentration. Later, and coincident with the first traces of skin darkening and necrosis, the CO<sub>2</sub> concentration rises still further and the curve of CO<sub>2</sub> *plus* O<sub>2</sub> cuts the 21 per cent. line. The last four or five estimations of O<sub>2</sub> concentration show some irregularity: as the pulp becomes semi-liquid in the final stages of senescence the tendency would appear to be one making for a more or less complete extinction of the O<sub>2</sub> concentration. (In some instances the slightly increasing oxygen concentrations observed at this time might have been due to leakage as a result of the sampling tube becoming slack, but evidence has been obtained which indicates that this is not the general explanation.)

Fig. 9 also shows the manometer record of pneumatic pressure within a fruit taken from the same row of the same hand and maintained under the

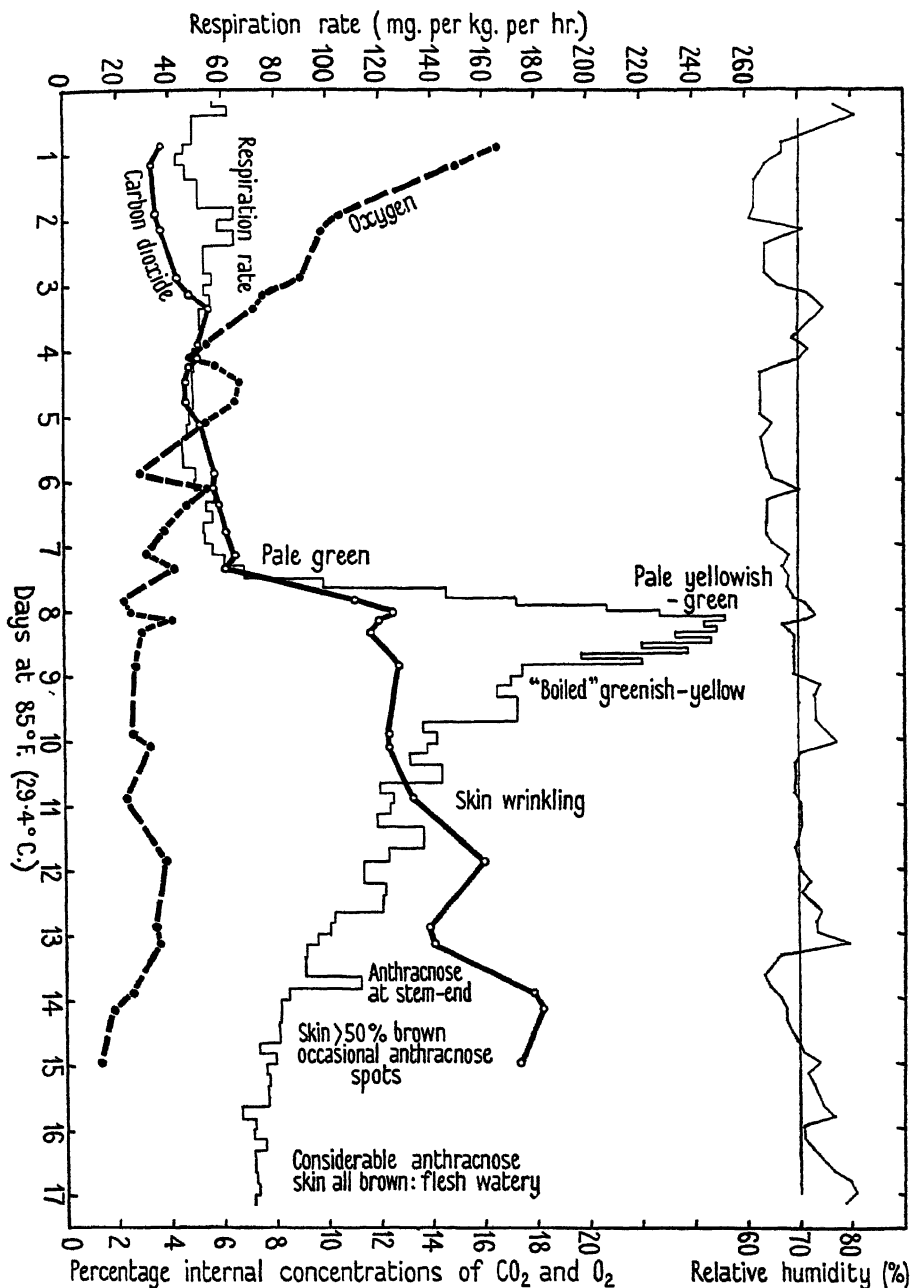


FIG. 7. Curves showing rate of respiration and internal concentrations of  $\text{CO}_2$  and  $\text{O}_2$  during the ripening of a heavy '3/4-full' fruit held at 85° F. (29.4° C.) and at low R.H.

same conditions. Allowing for small differences in the times at which ripening changes were initiated in the two fruits, it will be seen that the trend of the  $\text{CO}_2$  plus  $\text{O}_2$  curve is accompanied by a comparable trend in the curve of pneumatic pressure.

Fig. 10 illustrates data on internal gas concentrations in a 'heavy  $\frac{3}{4}$ -full',

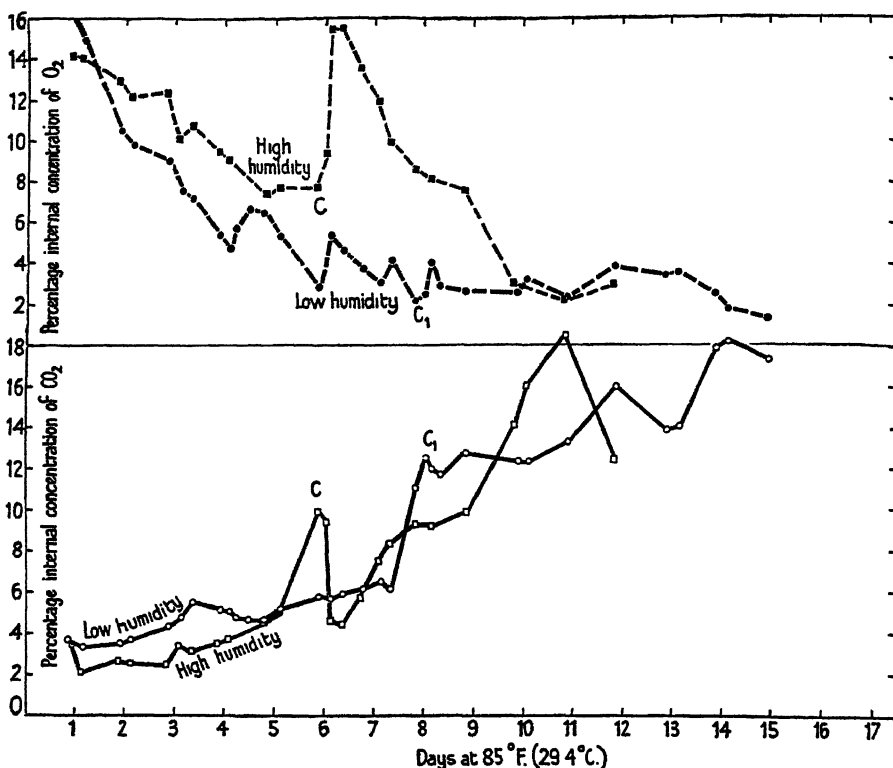


FIG. 8. Internal concentrations of  $\text{CO}_2$  and  $\text{O}_2$  during the ripening of two heavy ' $\frac{3}{4}$ -full' fruits held at 85° F. (29.4° C.) and at low (circles) and at high (squares) relative humidities. C, C<sub>1</sub>, climacteric peaks.

lower row fruit taken from the same hand as the fruit the respiration of which is shown in Fig. 7. At the climacteric the  $\text{CO}_2$  concentration rose to 9.23 per cent., and  $\text{O}_2$  declined to 3.03 per cent. Thereafter the characteristic recovery of  $\text{O}_2$  concentration took place, but it will be noted that the curve of  $\text{CO}_2$  concentration continued at approximately the same high level for some time, fell off slightly, then rose during final senescence. In respect of transpiration losses, this fruit occupied an intermediate position between the fruits held at high and low relative humidities illustrated in Figs. 2 and 7. This type of curve has frequently been obtained in similar experiments. In a second fruit from the same (lower) row, the internal  $\text{CO}_2$  reached a concentration of 10.69 per cent. at approximately the climacteric peak, while the  $\text{O}_2$  curve, having

descended to a low value, failed to show the typical post-climacteric recovery, being in this respect comparable to the fruit the behaviour of which is illustrated in Fig. 7.

An important and outstanding feature of the data obtained in these studies

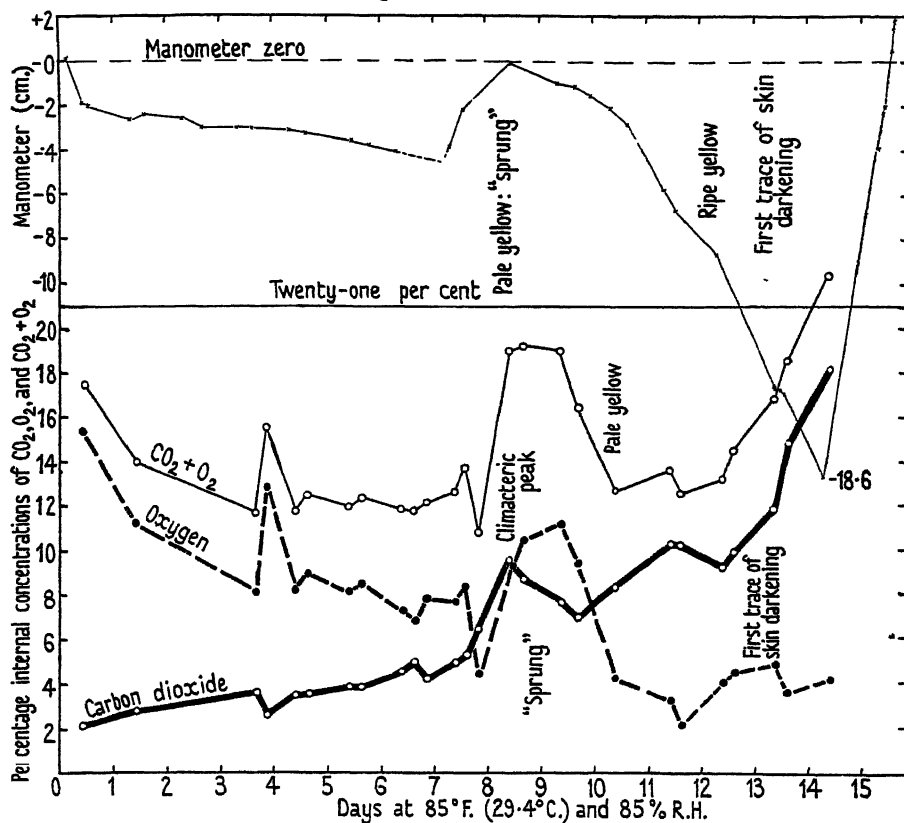


FIG. 9. Internal concentrations of  $\text{CO}_2$ , of  $\text{O}_2$ , and of  $\text{CO}_2$  plus  $\text{O}_2$  during the ripening of a '¾-full' fruit at  $85^\circ\text{F}$ . ( $29.4^\circ\text{C}$ .) and 85 per cent. R.H. The record of manometric pressure (cm. of Brodie's sol.) in a comparable fruit from the same 'hand' is also shown.

is the low values to which the internal concentration of  $\text{O}_2$  may descend as the climacteric peak of  $\text{CO}_2$  liberation is being approached. In Fig. 11 data showing trends of internal oxygen concentration for a group of 'heavy ¾-full' fruits taken from the same hand are set out. It will be realized that various circumstances<sup>1</sup> make it difficult to select for an observation the precise time at which maximal  $\text{CO}_2$  and minimal  $\text{O}_2$  values are reached, but in Fig. 11 it will be seen that some very low  $\text{O}_2$  concentrations were observed, e.g. 1.13, 1.29, 1.88 per cent., the subsequent recovery values in this lot of fruit being

<sup>1</sup> For a consideration of the limitations of the method employed see Wardlaw and Leonard 1939).



also low. In other experiments  $O_2$  concentrations as low as 0.3 per cent. have been obtained at the climacteric. The rate of attainment of these low  $O_2$  concentrations indicates that during the initiation of the climacteric phase its rate of utilization in metabolic processes must have increased. The trend

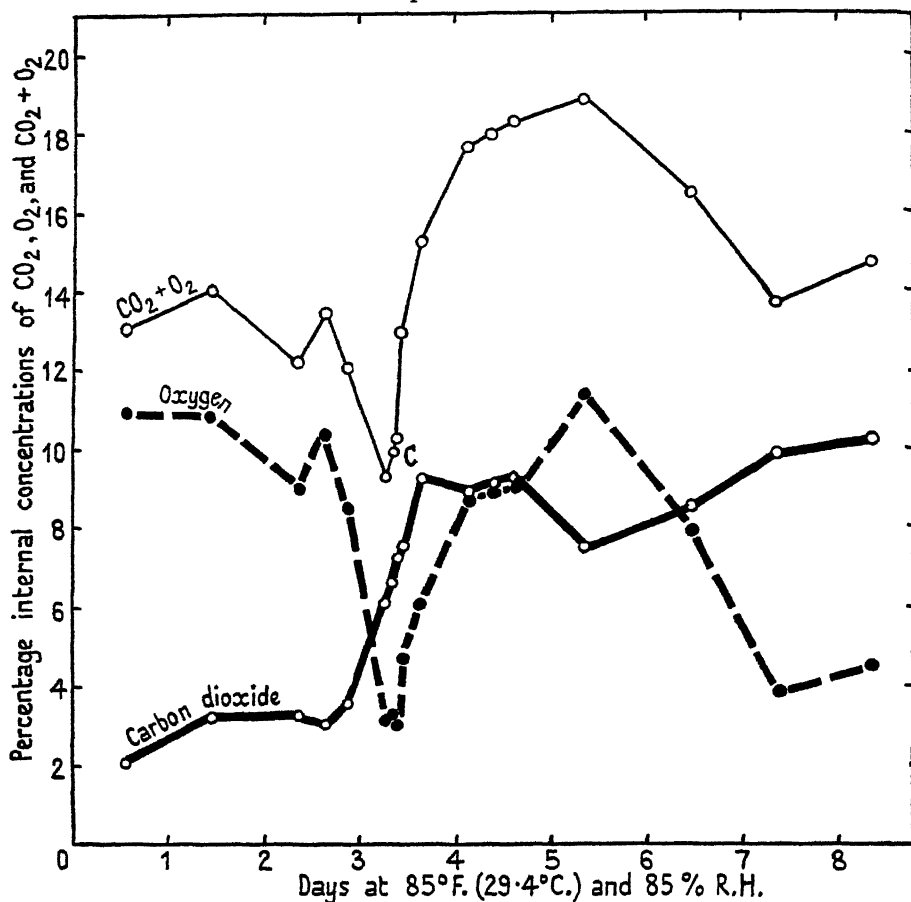


FIG. 10. Internal concentrations of  $CO_2$ , of  $O_2$ , and of  $CO_2$  plus  $O_2$  during the ripening of a 'heavy  $\frac{3}{4}$ -full' lower row fruit at 85° F. (29.4° C.) and 85 per cent. R.H. In this and similar fruits a typical  $CO_2$  climacteric peak was not observed.

towards the extinction point also introduces a factor which should be taken into consideration in the analysis of the climacteric phase.

The foregoing data give an indication of the existence, in different grades of fruit produced under different cultural conditions, of considerable variation in internal gas concentrations and by inference in metabolic processes during ripening.

The effect on the trend of internal gas concentrations which results from severing the fingers from the hand has also been investigated. Sampling tubes

were inserted into two top-row fingers remaining on the hand and into two adjacent fingers which had been cut off. Except for the slight variations already known to occur from finger to finger, all the main phases of ripening, as denoted by the changing trends of internal concentrations of  $\text{CO}_2$  and  $\text{O}_2$ , were closely comparable in attached and detached fingers.

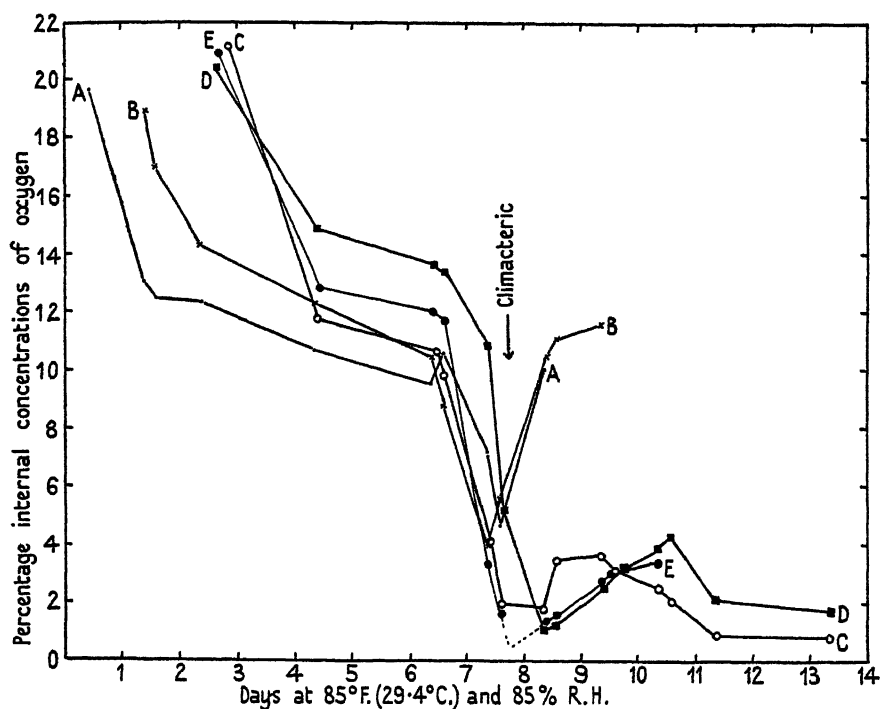


FIG. 11. Internal concentration of  $\text{O}_2$  during ripening at 85° F. (29.4° C.) and 85 per cent. R.H. in five '¾-full' fruits taken from the same 'hand'.

(b) *Regional distribution of internal gas concentrations.*

In view of the fact that ripening takes place regionally from the central pulp outwards to the skin (Wardlaw and Leonard, 1939) and that tissues offer resistance to the movement of gases, it may be anticipated that different concentrations of  $\text{CO}_2$  and  $\text{O}_2$  will be present in different positions.

A comparison of internal gas concentrations during the ripening of a '¾-full' fruit is shown in Fig. 12, the fruit having been supplied with gas-sampling tubes at both the proximal and distal ends. During the pre-climacteric phase, which lasted seven days, the close similarity of records, both in respect of  $\text{CO}_2$  and  $\text{O}_2$  concentrations, will be noted. With the onset of ripening during the seventh day, both the  $\text{CO}_2$  and  $\text{O}_2$  records indicate that the respiration changes associated with the climacteric were first observable at the distal end

and, a few hours later, at the proximal end. During the phase of recovery of oxygen concentration it will be noted that this is more marked at the tapering distal end, where the proportion of surface to volume is comparatively high,

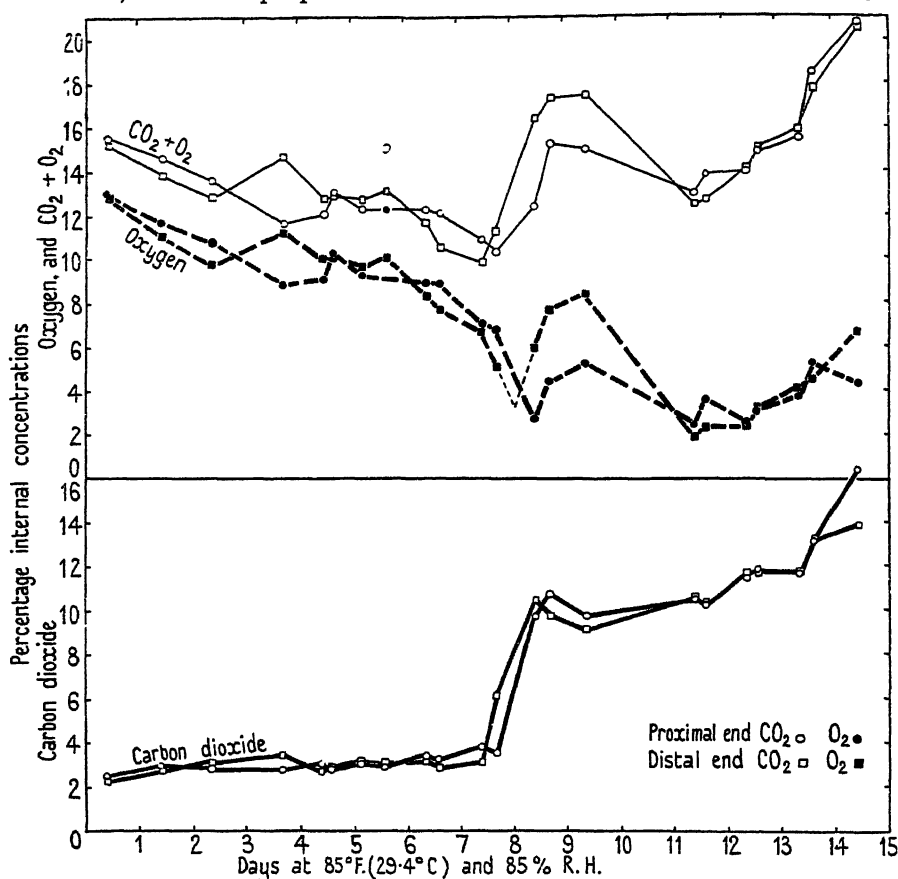


FIG. 12. Internal concentrations of  $\text{CO}_2$ , of  $\text{O}_2$ , and of  $\text{CO}_2$  plus  $\text{O}_2$  at the proximal (stem) and distal (stylar) ends of a '3/4-full' fruit. Ripening takes place slightly earlier at the distal than at the proximal end.

than at the more bulky proximal end, where it is lower. In the  $\text{CO}_2$  curve the descent from the climacteric peak value is slight in both positions and thereafter, with a high degree of uniformity, the two curves undergo the rise typical of late senescence.

At the termination of observations on the fourteenth day the fruit had not yet undergone the typical skin darkening of the final senescent phase; only the smallest trace of fungal activity was evident at the stem end, and the sampling tubes were still firmly inserted in the tissues. The rising  $\text{O}_2$  concentrations observed at both ends during this phase therefore deserve consideration. Although evidence has been obtained which indicates that at this

stage tissue resistance to the movement of gases is high and on the increase, nevertheless  $O_2$  in excess of that utilized in metabolism evidently passes through the outer tissues to the central region. An explanation that would appear to fit the observed facts is that during final senescence the process by which  $O_2$  is utilized has become disorganized: as a result the oxygen concentration shows a slow increase. This is in keeping with a view expressed by Kidd (1935) that during the senescence of apples there is a diminishing activity of the system for the oxidation of aldehyde by molecular oxygen.

Fig. 13 gives the data obtained when a '¾-full' fruit was provided with gas-sampling tubes (*a*) inserted in the placental region at the distal end, and (*b*) inserted transversely in a median position, so as to sample the sub-skin region, as illustrated by Wardlaw and Leonard (1939, Fig. 3 (iii) and (ii) respectively). It will be seen (i) that the climacteric changes in  $CO_2$  and  $O_2$  concentrations took place in the central tissues slightly in advance of the skin and sub-skin tissues, (ii) that the carbon dioxide concentration was consistently higher at the centre of the fruit than under the skin until the climacteric, and (iii) that the oxygen concentration was consistently lower at the centre than under the skin, except for a brief period during the post-climacteric recovery phase. It will be noted that after the climacteric the  $CO_2$  and  $O_2$  concentrations were higher under the skin than at the centre of the fruit. This is of interest in relation to the  $CO_2$  content of the pulp and skin observed during ripening (see section VI).

Fig. 14 shows the internal concentrations of  $CO_2$ ,  $O_2$ , and  $CO_2$  plus  $O_2$ , for another '¾-full' fruit, from the same hand as those of Figs. 9 and 13, the sampling tube being inserted in a median, sub-skin position. The several changes in gaseous concentration which accompany ripening are demonstrated, a triple crossing of the  $CO_2$  and  $O_2$  concentration curves again being seen. Reference will be made later to the manometer record in this figure.

In each fruit the tube insertion was inspected at the end of the experiment to ascertain whether cork formation, which would be inimical to the gas-sampling technique adopted, or other changes such as internal rotting of tissue, had taken place. Fungal rotting seldom occurred, but in some instances a small amount of cork formation was observed in the sampling tube bore, especially at the apical end, which may account for some of the temporary departures from the general trend shown in the curves during the pre-climacteric phase.

### (c) Trends in gas concentrations in anomalous fruit.

It has already been pointed out (Wardlaw, Leonard, and Barnell, 1939) that the maturity of a bunch cannot always be judged on the criterion of size alone. Thus a bunch developing under conditions of rank growth may rapidly attain to large size, and be harvested as 'heavy' grade fruit, though it is still relatively immature. Again, where particular growth conditions have been

such as to modify the texture of pulp and skin and the ratio of pulp to skin it may be anticipated that, by comparison with other fruit used in these experiments and selected as typical and standard, differences may be found in the

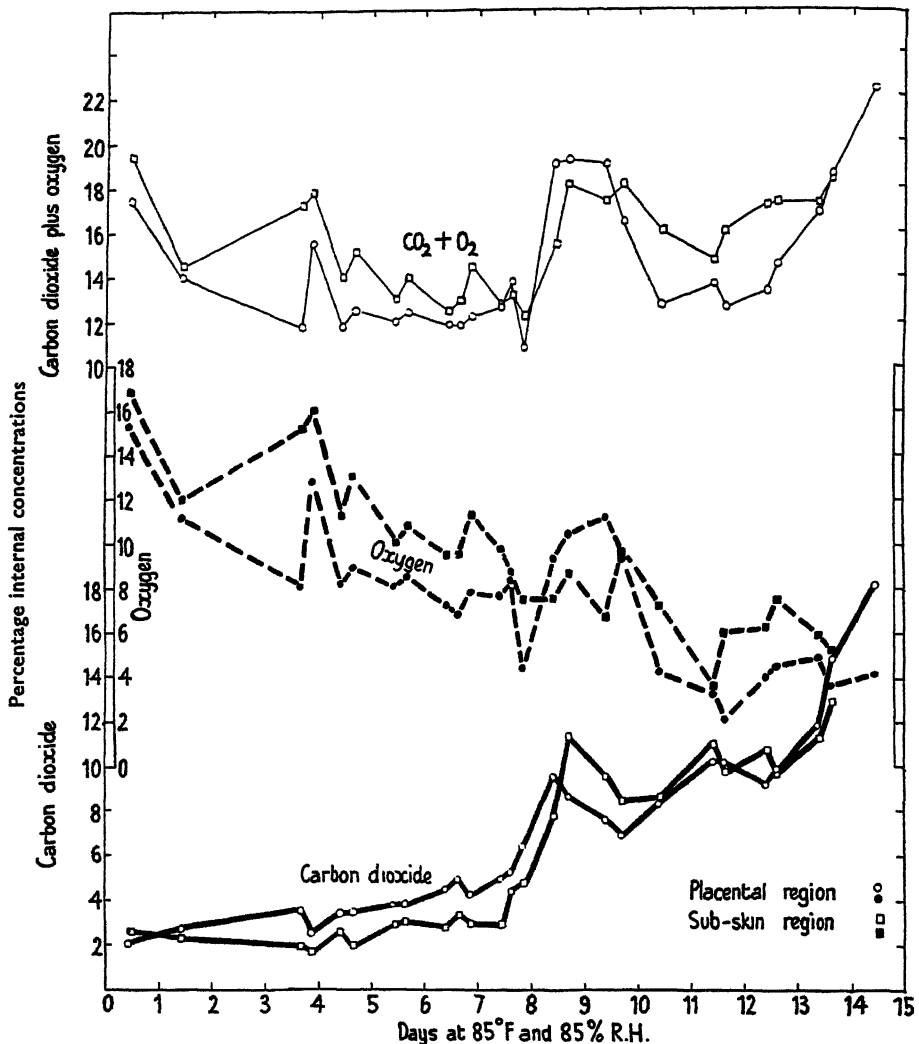


FIG. 13. Internal concentrations of  $\text{CO}_2$ , of  $\text{O}_2$ , and of  $\text{CO}_2$  plus  $\text{O}_2$  in the placental and sub-skin regions of a '3/4-full' fruit. Ripening takes place slightly earlier at the centre than in the region of the outer pulp and skin.

trends of internal gas concentrations during ripening. The data incorporated in Fig. 15, obtained from coarse fruit classed as 'heavy 3/4-full' at 85° F. and 85 per cent. relative humidity, illustrate this point. The fruit was provided with sampling tubes, inserted (a) at the stem end to penetrate the central placental region, and (b) in a median sub-skin position.

It will be noted that the pre-climacteric phase was unusually long, i.e. 9 days, as compared with 6–7 days for ‘ $\frac{3}{4}$ -full’ fruit and 3–5 days for ‘heavy  $\frac{3}{4}$ -full’ fruit. During this period the concentration of  $\text{CO}_2$  was slightly but

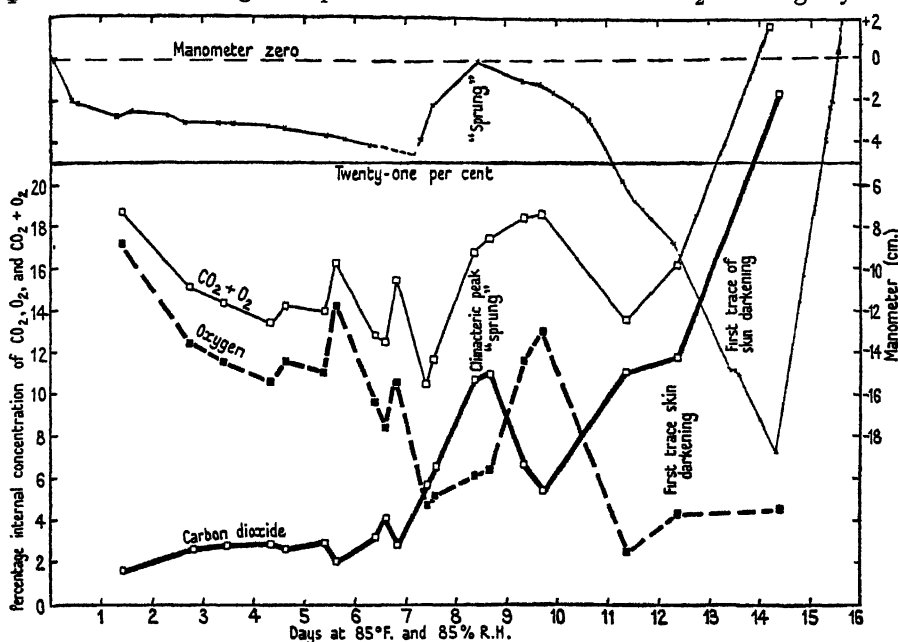


FIG. 14. Internal concentrations of  $\text{CO}_2$ , of  $\text{O}_2$ , and of  $\text{CO}_2$  plus  $\text{O}_2$  in a sub-skin position during ripening of a ‘ $\frac{3}{4}$ -full’ fruit at 85° F. (29.4° C.) and 85 per cent. R.H. The manometer record for a comparable fruit is also shown.

consistently higher at the centre than under the skin, the  $\text{O}_2$  concentration being low in both positions. The onset of the climacteric, though marked by the characteristic rapid rise in the internal concentration of  $\text{CO}_2$ , was accompanied by an inconsiderable decline in the concentration of  $\text{O}_2$ . The post-peak recovery in  $\text{O}_2$  concentration was also slight, and the decline in  $\text{CO}_2$  concentration from the peak value negligible. It is of interest that the sub-skin region showed climacteric changes slightly in advance of the central, placental region, other fingers from the same hand also providing evidence of early ripening of the outer pulp—a circumstance which has occasionally been encountered in these studies. Using the usual pressure criterion, it was particularly difficult to determine, in fruits of this series, when the ‘sprung’ condition had been reached—a possible reflection of anomalous tissue constitution.

## V. INTERNAL PRESSURES DURING RIPENING

### (a) Internal pressures in relation to respiration.

In the earlier sections the internal atmosphere in fruits has been expressed in terms of the percentage concentrations of  $\text{CO}_2$  and  $\text{O}_2$  as ascertained by the

Haldane gas-analysis apparatus. In view of the fact that the movement of gases during respiration takes place along pressure gradients it would have been desirable for these observations on internal gases to have been described in

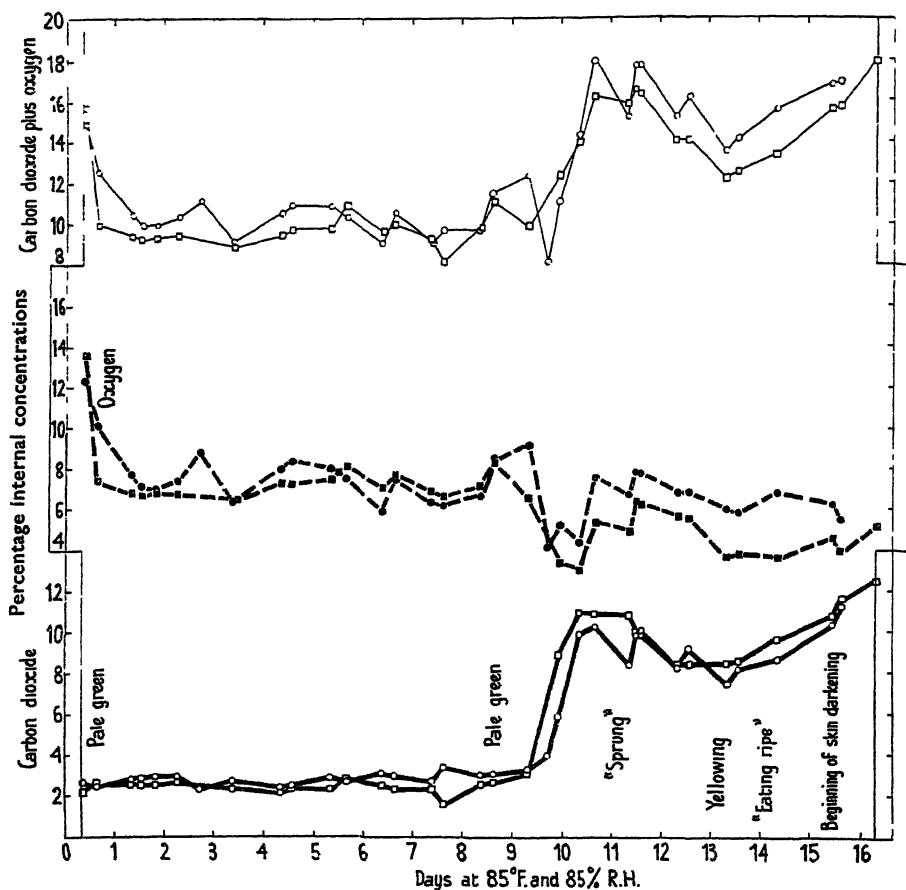


FIG. 15. Internal concentrations of CO<sub>2</sub>, of O<sub>2</sub>, and of CO<sub>2</sub> plus O<sub>2</sub> during the ripening at 85° F. and 85 per cent. R.H. of an anomalous fruit: circles, at proximal (stem) end; squares, in sub-skin position.

Although selected as a 'heavy  $\frac{3}{4}$ -full' fruit because of its size and weight it did not show the climacteric rise until the ninth day, the CO<sub>2</sub> peak value being reached in the sub-skin position earlier than in the central placental region.

terms of partial pressures. This, however, has been precluded by the methods adopted, no cognizance having been taken of the partial pressure of water vapour in each estimation. In the present section further experimental data are presented which involve actual pressures, i.e. the sum of the partial pressures of all gases present.

In an earlier paper (Wardlaw and Leonard, 1938) it was shown that by attaching sensitive manometers to several different tropical fruits considerable

departures from the normal atmospheric pressure could be demonstrated during development and ripening. Examination of the manometer records obtained showed that the definite trends observed could be used in the elucidation of respiration phenomena, particularly in respect of tissues whose resistance to the movement of gases was undergoing change. The manometer record, in fact, gives the differences between the sum of the partial pressures of the gases present within a fruit and in its external atmosphere at each of the several phases of ripening and senescence.

Fruits from the same hand and comparable with those used in respiration studies were fitted with manometers as described in an earlier paper (Wardlaw and Leonard, 1939), and kept under observation in the same storage room as the other respiration experiments. In Figs. 9 and 14 a manometer record is shown in conjunction with the curves of internal gas concentrations obtained. The close similarity in the general trends of the manometer curve and that for the sum of the concentrations of  $\text{CO}_2$  and  $\text{O}_2$  is at once apparent. In particular the phase of decreased  $\text{O}_2$  concentration at the climacteric, the subsequent recovery and decline in that concentration and the increasing concentration of  $\text{CO}_2$  during late senescence are all represented by changes in trend in the manometer record.

Such observations based on different fruits, even when these have been taken from the same hand, are not sufficient to show the precise relationship in time between the more marked changes in pneumatic pressure and respiration. To establish this relationship, measurements of respiration rate were made for a fruit placed in a respiration chamber as already described (Wardlaw and Leonard, 1939), both the chamber and the fruit being provided with similar manometers. As the atmosphere in the respiration chamber in a draw-through system is at a slightly reduced pressure, the actual pneumatic pressure within the fruit at any time is given by the difference between the two manometer readings.

Fig. 16 shows, for a 'heavy  $\frac{3}{4}$ -full' fruit at  $85^\circ \text{F}$ . and 100 per cent. relative humidity, the curve of respiration rate and of internal pneumatic pressure obtained by the above method. In this fruit the onset of the climacteric took place almost immediately after the commencement of the experiment. It will be seen that the manometer record shows a well-marked decrease in pressure which coincides precisely in time with the onset of the climacteric rise, i.e. with the time at which the concentration of  $\text{O}_2$  undergoes a marked decrease, as also does the sum of the concentrations of  $\text{CO}_2$  and  $\text{O}_2$ . After the climacteric peak the pneumatic pressure is seen to be approximately that of the surrounding atmosphere. But later in the senescence of the fruit, about the time when anthracnose spots are beginning to appear, a decline in pneumatic pressure again takes place. This coincides with a decline in respiration rate and also with a downward trend towards the extinction point of the internal concentration of oxygen. At this stage the soft ripe tissues offer increased resistance to the movement of gases, the evidence for this being



that while the respiration rate is decreasing the internal concentration of  $\text{CO}_2$  is increasing. The next ripening change is such that the manometer shows a sharp rise, positive pressures within the fruit being recorded. But

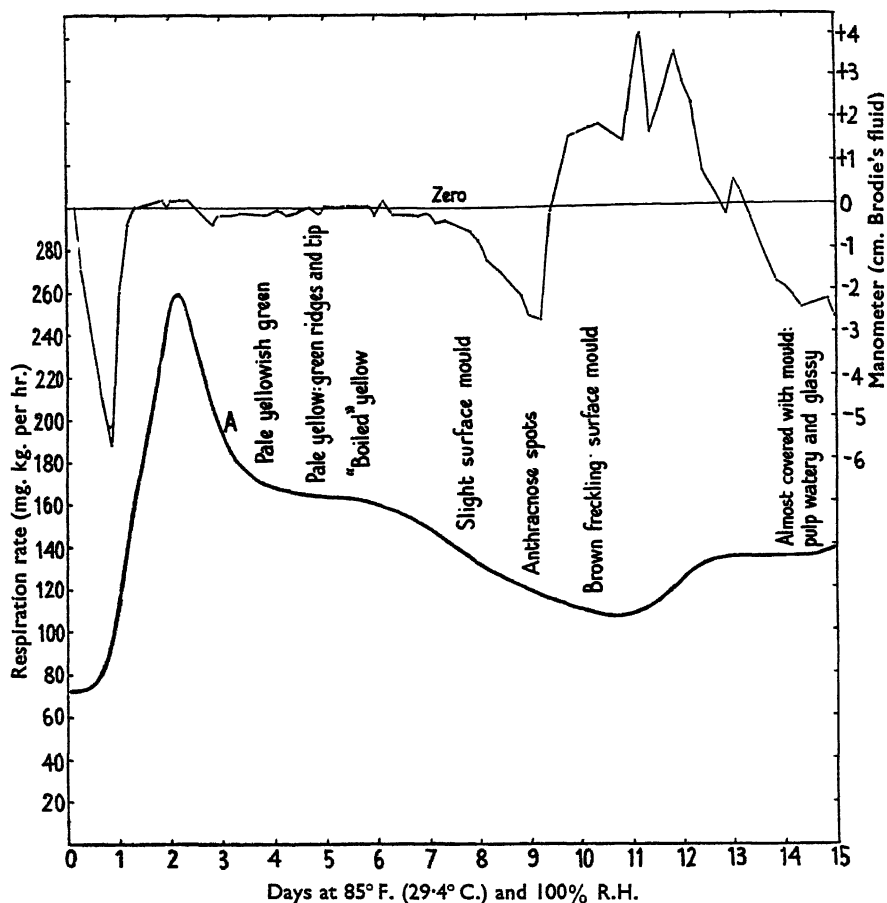


FIG. 16. Curve of respiration rate and manometer record obtained from the same fruit during the ripening of a 'heavy  $\frac{3}{4}$ -full' Gros Michel fruit at 85° F. (29.4° C.) and 100 per cent. R.H. At point A the manometer attached to the fruit ceased to show slight fluctuations in response to fluctuations in the pressure in the respiration chamber—an indication that the 'sprung' condition had been attained.

whereas this stage is characterized by only a slight increase in the respiration rate, it has been ascertained that the internal concentration of  $\text{CO}_2$  rises rapidly, values approaching and even exceeding 21 per cent. having been obtained. As the concentration of  $\text{O}_2$  may also show a slow augmentation at this stage, the manometer record of positive pressure is again seen to be closely correlated with ascertained facts concerning the sum of internal gas

concentrations. During final senescence a trend towards negative pressure was again recorded; this is discussed in the next section.

(b) *Effect of transpiration.*

A comparison of the manometer curves illustrated in Figs. 9 and 16 indicates that whereas in the former, obtained from a fruit at 85 per cent. relative humidity and therefore transpiring comparatively rapidly, the curve for the greater part lies on the negative side of the zero line, in the latter, obtained from a fruit at 100 per cent. relative humidity, with a slow rate of transpiration, the curve for a considerable period approximately coincides with the line of zero pressure difference. The rate at which transpiration is proceeding is thus seen to be one of the factors concerned in determining the pneumatic pressure in fruits. Again in Fig. 9, where two comparable fruits were used (i.e. one for observation of internal gas concentrations and one for pneumatic pressure), it will be seen that although both reached the 'sprung' condition at almost identically the same time, the curve of the concentrations of ( $\text{CO}_2$  plus  $\text{O}_2$ ) cuts the 21 per cent. line considerably in advance of the point at which the manometer curve cuts the line of zero pressure difference. Moreover, the lowest negative pressure observed on the fourteenth day appears to be disproportionately low by comparison with the lowest sum value of  $\text{CO}_2$  and  $\text{O}_2$  observed on the eleventh day, or with the pre-climacteric trough value on the eighth day. In brief it would appear that transpiration tends to create a negative pressure in fruits, particularly during the later stages of senescence.

To investigate this aspect more fully, two comparable ' $\frac{3}{4}$ -full' fruits were fitted with manometers and placed in two storage rooms maintained at a temperature of 85° F., but with relative humidities of 85 and 55 per cent. respectively. The manometer records illustrated in Fig. 17 show that both fruits passed through the major ripening phases at approximately the same time. The decrease in negative pressure during the climacteric was less marked in the fruit at low relative humidity. During late senescence important differences between the two records of manometric pressure became apparent. For whereas the fruit at the higher humidity showed a decrease in negative pressure and eventually a positive pressure, the fruit at the lower humidity showed only a brief trend during approximately thirty-six hours in the direction of decreasing negative pressure, after which the downward trend of the manometer curve was resumed so that very low pressures were recorded. As it has been demonstrated that there is a marked increase in the internal concentration of  $\text{CO}_2$  during late senescence, in fruits at both high and low humidity, it may be inferred that the differences between the two curves of manometric pressures shown in Fig. 17 are a direct result of differences in transpiration.

Fig. 18 shows the manometer record for a banana fruit at 85° F. and 85 per cent. relative humidity. (At the beginning of the experiment the drop

in manometer pressure coincided with a decline in the internal concentration of  $O_2$  observed in other fruit from the same hand, Fig. 13.) After remaining at a steady, slightly negative pressure for four days the curve shows the following

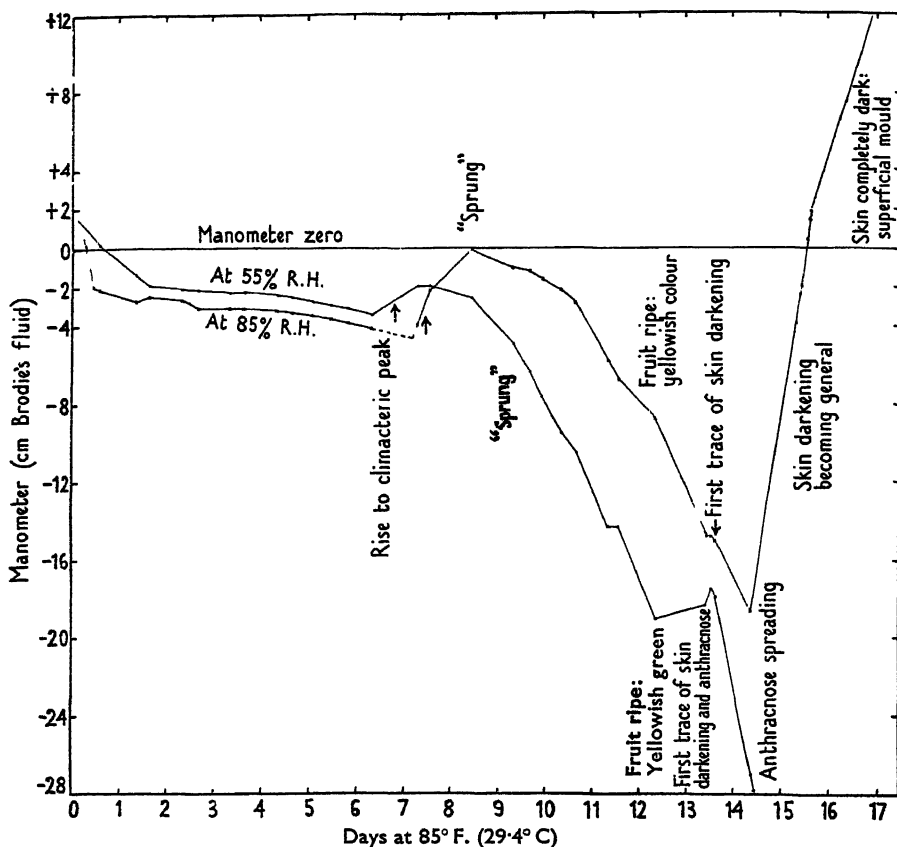


FIG. 17. Manometer records obtained during the ripening of two comparable '¾-full' fruits held at 85° F. (29.4° C.). The fruits were maintained at relative humidities of 55 and 85 per cent. The general similarity of the curves up to the ripe stage and the marked dissimilarity thereafter will be noted. See text.

successive changes: a typical downward trend at the onset of the climacteric followed by a period of partial recovery; a steep downward trend coinciding in time with the trend of  $O_2$  concentration towards the extinction point during the post-climacteric period; a recovery to zero pressure difference with the onset of final senescence, denoted by the darkening of the skin; and a final phase in which very considerable negative pressures developed, the latter being associated in time with the desiccation and shrivelling of the skin and the liquefaction of the pulp.

The negative pressure observed in fruits at low humidity, particularly

during final senescence, would appear to be referable to the effect of transpiration or evaporation in any aqueous system, whether biological or purely physical.

In a biological system the extent to which the reduction in pressure will be diminished will be governed by (a) the resistance of the tissues to the movement of gases, (b) the extent to which gases ( $\text{CO}_2$ ,  $\text{O}_2$ , and  $\text{N}_2$ ) retained in the

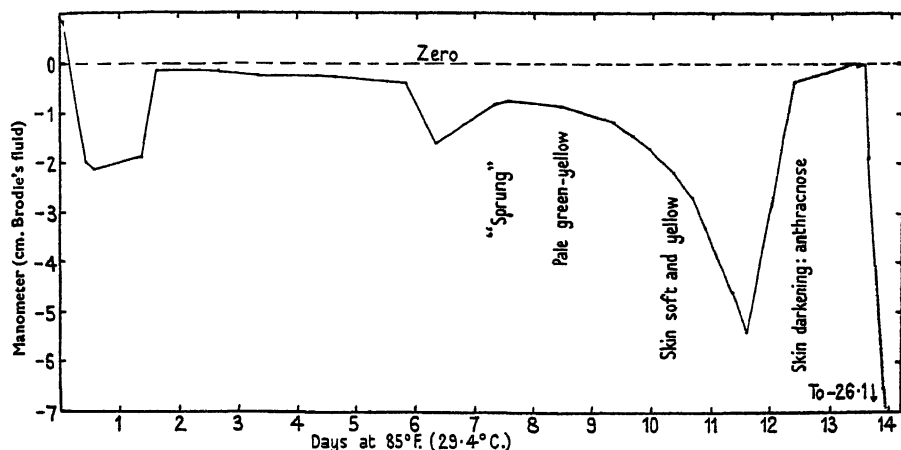


FIG. 18. Manometer record obtained during the ripening of a 'heavy  $\frac{3}{4}$ -full' fruit at 85° F. and 85 per cent. R.H. See text.

tissues can come out of solution, and (c) the pressure of water vapour in the intercellular air spaces. During late senescence resistance to the inward movement of gases will be offered by: (i) the occlusion of air passages as the result of tissue shrinkage, distortion, or partial liquefaction, with concomitant restriction of the diffusive or mass movement of gases, and (ii) the reduction of water content, in particular that of the epidermis and sub-epidermal tissues, whereby the movement of gases in solution will be affected. That changes in the several tissues do take place during the later stages of senescence, whereby the factors discussed above become operative, is known from inspection and is being made the subject of anatomical and histological investigations.

Although during late senescence the tissue content of  $\text{CO}_2$  is high (see section VI), nevertheless this gas does not come out of solution in sufficient quantity to offset the trend in negative pressure created by transpiration.

Questions pertaining to the movement of gases and pneumatic pressures within tissues have been considered at some length by Pfeffer (1899). The following observations are of special interest in relation to the data presented here: as a cell-wall dries, i.e. as its percentage content of water decreases, its permeability to gases also decreases; as the mass movement of gases is more rapid than their 'diostotic' movement, i.e. in solution through membranes, the presence of open channels facilitating gaseous movement is important;

where the intercellular air-space system remains open, it is unusual to find well-marked negative pressures. Reference may also be made to a recent paper by Curtis (1937) who shows that, *inter alia*, temperature differences produce pressure differences on opposite sides of a porous membrane.

(c) *Comparison with other fruits.*

With the exception that in large green papaws small positive pressures may be recorded prior to the onset of ripening—a phenomenon for which some explanation has been adduced in a previous paper (Wardlaw and Leonard, 1938)—the major features of the manometer records obtained during the ripening of bananas are in all respects in conformity with those for the papaw in that the manometer affords an expression of the differences between the sums of the partial pressures of the gases present inside and outside the fruit.

It is not improbable that the use of the manometer in metabolic studies of fruit can be still further extended. The writers have in mind the essential contrasts that must exist in respect of all gaseous phenomena, including transpiration, between large spherical or sub-spherical fruits such as the papaw or water-melon where the proportion of surface to volume is very small, and small cylindrical fruits such as the banana where the proportion of surface to volume is comparatively high. Moreover, tissue texture differences and, in the banana, pulp/skin ratios are also involved, these being important both on theoretical and practical grounds.

It is also known that during the climacteric a rise in temperature and a rapid increase in transpiration (Leonard, 1940) take place. To what extent these factors exercise an influence on the manometer record is not known but is being examined as part of the general investigation of water relations during ripening.

## VI. CARBON DIOXIDE CONTENT OF TISSUES

(a) *Method and materials.*

In these studies an attempt has been made to ascertain the relationship between the internal concentration of  $\text{CO}_2$  and the amount of that gas retained in the tissues. The method<sup>1</sup> of determining the  $\text{CO}_2$  content of a tissue has already been described (Wardlaw and Leonard, 1939). As each estimation involves the destruction of a fruit, and, further, as it is desirable to sample upwards of twelve fruits during the ripening period, the requisite number was obtained by selecting fingers from the upper rows of two adjacent hands. Material so obtained exhibits a high degree of uniformity.

<sup>1</sup> The determination of the  $\text{CO}_2$  content of tissue by the method used is still being made the subject of investigation. As it is not known in what form  $\text{CO}_2$  is present in tissues, the values submitted may be provisionally described as being those for the  $\text{CO}_2$  present as dissolved gas or derived from compounds readily yielding this gas on boiling in alcohol. The possibility that small quantities of organic acid distil over is also being considered.

Fruits were cut off from the hands, all at the same time, weighed, provided with gas-sampling tubes, placed in a room maintained at 85° F. and 85 per cent. relative humidity, and the internal concentrations of CO<sub>2</sub> and O<sub>2</sub>

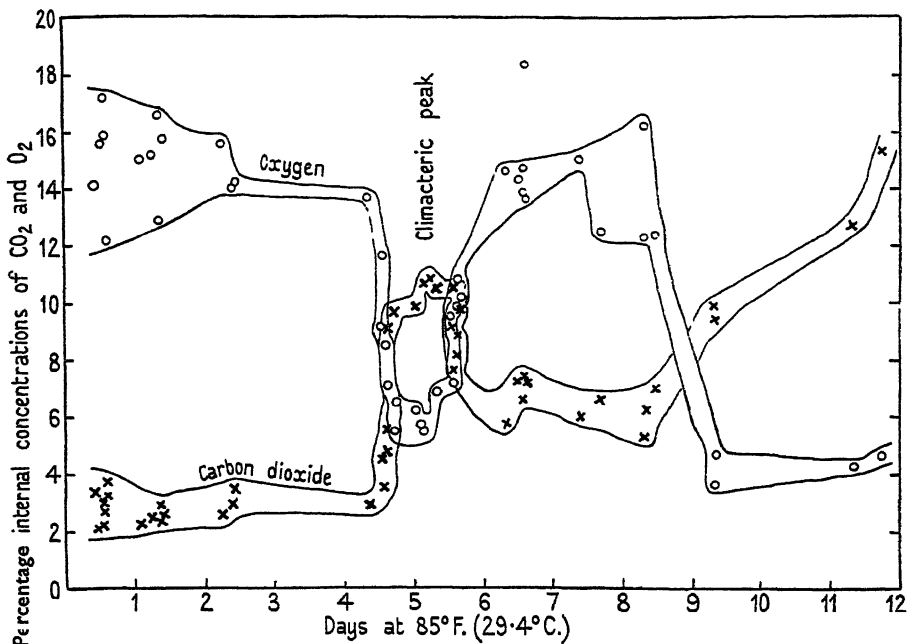


FIG. 19. Record of internal concentrations of CO<sub>2</sub> and of O<sub>2</sub> obtained during ripening at 85° F. (29.4° C.) and at 85 per cent. R.H. of several '¾-full' fruits taken from one 'hand'. The general trends established by these observations are in agreement with those already illustrated for single fruits.

measured at regular intervals. Fruits were then selected from time to time for the estimation of the CO<sub>2</sub> content of tissues, knowledge of the trends of internal gas concentrations in individual fingers being used in the selection of a particular fruit at a particular time. Accordingly the curves for internal gas concentrations presented in conjunction with the curve of the CO<sub>2</sub> content of tissues are synthesized from an assemblage of records from different fruits so as to conform closely to the time-value sequence observed in a single fruit from the same source. With this explanation, the general trends established may be regarded as representative both in time and magnitude.

As an example of the degree of uniformity of material Fig. 19 shows an assemblage of all the records of internal CO<sub>2</sub> and O<sub>2</sub> concentrations obtained in the course of an experiment of this kind using '¾-full' fruit. The general trends of internal gas concentrations during ripening are in conformity with those already discussed and illustrated, the variability between fingers being indicated. The diminution in the number of points in the latter part of the

graph is due to the progressive utilization of fruits for determinations of the  $\text{CO}_2$  content of tissues.

(b) *Presentation of data.*

Fig. 20 shows (A) the trend of internal  $\text{CO}_2$  concentration during ripening, (b) the total  $\text{CO}_2$  content of fruit (calculated on the basis of a 150 gm. fruit),

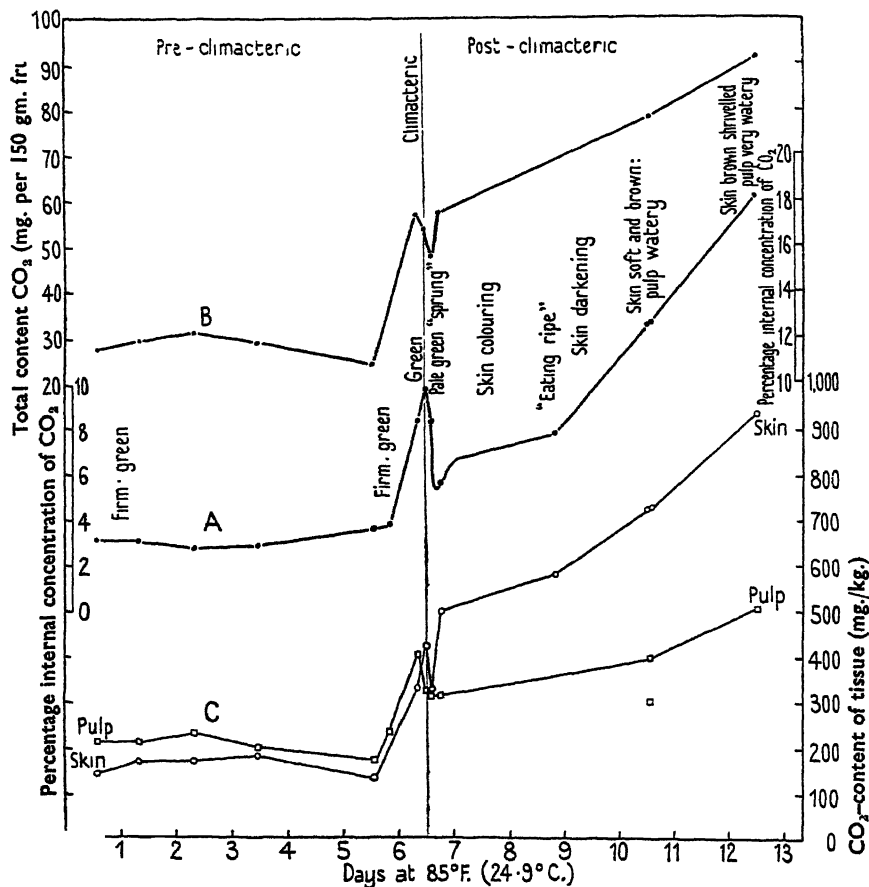


FIG. 20. Curves showing (A) the trend of internal concentration of  $\text{CO}_2$  during ripening, (b) the total  $\text{CO}_2$  content of fruits (calculated on the basis of a 150 gm. fruit), and (c) the  $\text{CO}_2$  content of pulp and skin (expressed as mg. per kg.).

and (c) the  $\text{CO}_2$  content of pulp and skin (as mg. per kg.) in fruits graded as 'heavy  $\frac{3}{4}$ -full'. For reference, the complete record of observations and calculations on which these graphs are based is shown in Table I. (below).

The form of the curve of internal  $\text{CO}_2$  concentration is in agreement with those already presented. The similarity between this curve and that of the total  $\text{CO}_2$  content of tissues will be noted. It is evident that at the climacteric changes take place whereby a new high level of  $\text{CO}_2$  content of tissues is

established and maintained during the post-climacteric period. In earlier sections it has been suggested that the form of the  $\text{CO}_2$  curve (whether of respiration rate or internal concentration) during the climacteric is to some extent the record of a transition effect: further evidence supporting this view is afforded by the rise and fall in the  $\text{CO}_2$  content of tissue. The curves, Fig. 20 C, indicate that in respect of the  $\text{CO}_2$  content of the pulp and skin a constant relationship is maintained during the pre-climacteric phase, the pulp containing a slightly but consistently greater quantity of  $\text{CO}_2$  per unit weight. The onset of the climacteric is marked by a rapid increase in the  $\text{CO}_2$  content of the pulp, this shortly preceding corresponding changes in the skin. The  $\text{CO}_2$  content of the pulp rises to a maximum value which precedes the peak of internal  $\text{CO}_2$  concentration, and also the peak value of the skin  $\text{CO}_2$  content. Both pulp and skin contents then fall from the peak value to a lower level, but whereas thereafter the pulp content rises slowly, the skin content rises rapidly and continues to rise, high values being maintained during final senescence. Further, it will be noted that subsequent to the climacteric peak, the curves of pulp and skin  $\text{CO}_2$  content show a crossing over. This observation is in conformity with the observations on regional ripening already described (section IV (b)). The post-climacteric rise in the  $\text{CO}_2$  content of the skin is closely associated with the colouring and softening processes which have been observed.

In the post-climacteric phase, the high  $\text{CO}_2$  content of tissues, while associated with a high internal gas concentration, is accompanied by a comparatively low  $\text{CO}_2$  liberation rate. The elucidation of the factors involved will obviously call for extended investigation.

Figs. 21 and 22 present further data obtained from '¾-full' fruit at 85° F. and 85 per cent. relative humidity, during a ripening period lasting for fifteen days. (Although these fruits were of approximately the same average weight as those used in the experiment described above, the actual maturity was that of '¾-full' fruit.)

In Fig. 21 the data on internal gas concentrations obtained during the pre-climacteric phase have been treated to show (a) average daily values for  $\text{CO}_2$  and  $\text{O}_2$ , and (b) the range in such values. From this it will be seen that there is a slow upward trend in  $\text{CO}_2$  concentration and a slow downward trend in  $\text{O}_2$  concentration as the climacteric is approached. The curve of  $\text{CO}_2$  plus  $\text{O}_2$  concentration (Fig. 22 A) also shows a slightly downward trend.

Fig. 22 shows (A) the internal concentrations of  $\text{CO}_2$ ,  $\text{O}_2$ , and ( $\text{CO}_2$  plus  $\text{O}_2$ ) of the individual fruits selected in sequence for the determination of the  $\text{CO}_2$  content of skin and pulp during ripening; (B) the changes in the total content of  $\text{CO}_2$  based on a fruit weighing 150 gm.; (c) the  $\text{CO}_2$  content as mg. per kg. in skin and pulp, and (d) the ratio of weight of pulp to that of skin. Fig. 22 D indicates that the climacteric is marked by definite changes in the weight relationship of skin and pulp: the comparatively high and low sugar contents of pulp and skin respectively, observed once ripening changes have been initiated, suggest a physiological mechanism which would account



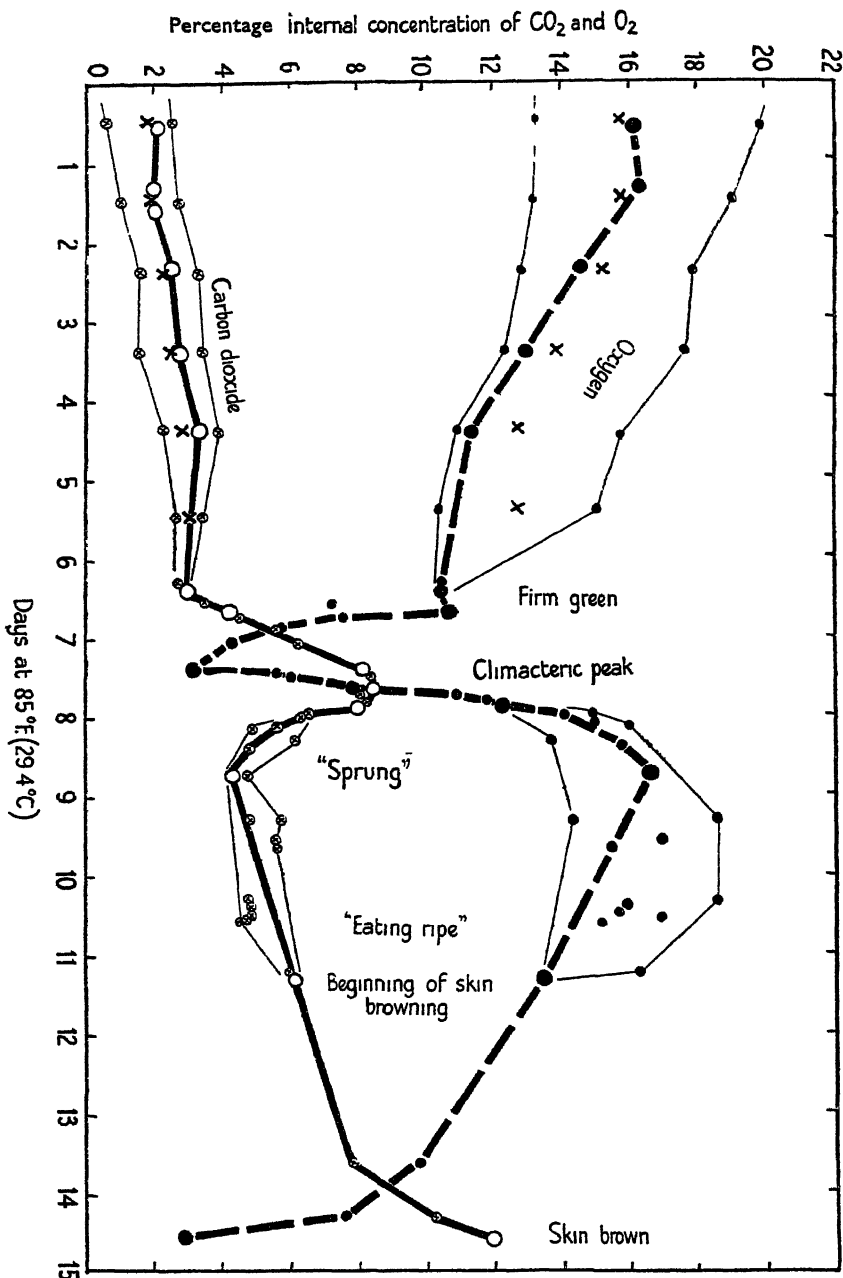


FIG. 21. Collective data on internal concentrations of  $\text{CO}_2$  and of  $\text{O}_2$  in upper row 'fingers' from the third and fourth 'hands' of a  $\frac{3}{4}$ -full' bunch, during ripening at 85° F. (29.4° C.) and 85 per cent. R.H. The heavy lines indicate the trend of internal concentrations of  $\text{CO}_2$  and  $\text{O}_2$  during ripening; the large circles denote the individual fruits used for the estimations of the  $\text{CO}_2$  content of the tissue. During the pre-climacteric phase the range in gas concentrations and the mean values (large crosses), each based on 8-14 readings, are shown. All the data obtained during the post-climacteric phase are also indicated. (See also Fig. 22.)

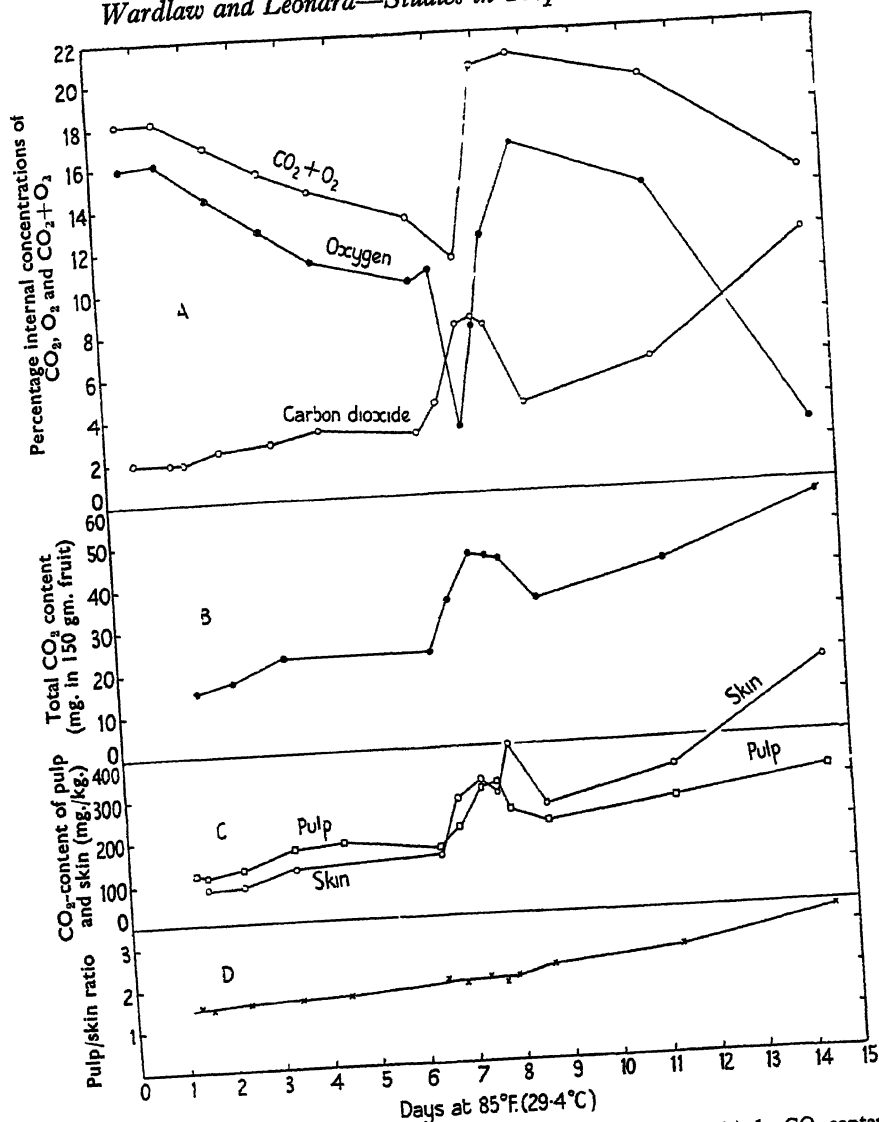


FIG. 22. Curves showing (A) the trend of internal gas concentrations, (B) the  $\text{CO}_2$  content of fruits (based on a 150 gm. fruit), (C) the  $\text{CO}_2$  content of pulp and skin (expressed as mg. per kg.), and (D) the pulp/skin weight ratio during the ripening of '2-full' fruits at  $85^\circ\text{F}$ . and 85 per cent. R.H. (See also Fig. 21.)

for the removal of water from skin to pulp. Leonard (1940) has shown that during this period transpiration is proceeding at a higher rate. He has pointed out that the whole of this subject deserves careful investigation as an aspect of the general metabolic changes in progress.

TABLE I

	Data of CO <sub>2</sub> content of tissue during ripening.													
No. of fruit	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Original weight	142.8	149.5	145.0	146.0	157.8	133.0	153.0	145.2	155.5	144.0	144.0	140.2	138.5	132.8
Weight at sampling	140.0	145.0	138.2	139.0	147.2	127.4	139.3	133.5	144.4	132.2	129.0	123.5	121.0	107.5
Percentage loss in weight	1.97	3.01	4.70	4.80	6.70	4.20	8.95	8.00	7.10	8.20	10.50	11.90	12.60	19.00
Weight of skin	56.7	54.5	52.5	53.1	51.8	47.9	45.4	44.1	52.0	44.9	36.0	34.5	30.0	25.5
Weight of pulp	82.6	89.8	84.2	84.8	94.2	78.4	92.4	88.8	92.1	85.5	92.2	88.5	90.2	80.2
Ratio pulp skin	1.46	1.65	1.60	1.60	1.81	1.64	2.04	2.00	1.77	1.90	2.56	2.56	3.01	3.14
Concentration internal CO <sub>2</sub>	3.10	3.15	2.79	2.87	3.62	3.78	8.31	9.68	8.36	5.62	7.71	12.52	12.57	18.11
CO <sub>2</sub> in skin (mg.)	8.14	9.32	8.93	9.71	7.06	*	14.91	18.64	17.17	22.57	20.90	25.12	21.89	23.94
CO <sub>2</sub> in pulp (mg.)	17.71	19.14	19.43	16.97	16.39	19.53	37.29	29.05	28.95	27.28	*	26.69	35.72	40.71
Total CO <sub>2</sub> (mg.)	25.85	28.46	28.36	26.68	23.45	—	52.20	47.69	46.12	49.85	—	51.80	57.61	64.65
CO <sub>2</sub> in 150 gm. fruit (mg.)	27.84	29.58	31.13	29.02	24.08	—	56.83	53.78	48.00	37.35	—	63.19	70.82	71.75
CO <sub>2</sub> in skin as mg./kg.	143.6	171.0	170.0	182.9	136.4	—	328.5	422.7	330.2	502.7	580.6	728.1	729.5	939.1
CO <sub>2</sub> in pulp as mg./kg.	214.4	213.0	230.8	200.4	174.0	249.1	403.5	327.1	314.3	319.0	—	301.6	396.0	507.7

\* Spoilt readings.

## VII. TEMPERATURE CHANGES OF TISSUES DURING RIPENING

The question of temperature changes during the ripening of fruit has already been discussed (Wardlaw and Leonard, 1939 and section III of this paper). It has been pointed out that the tissue temperature of a respiring fruit is not necessarily that of the surrounding air and may be modified according to the phase in maturation reached by the fruit, and by the temperature, humidity and rate of movement of the surrounding air.

To complete the present survey it will be enough to indicate that in bananas, ripened at tropical temperatures, the changes in the rate of heat production, and, as a result, in the temperature of tissues, are sufficiently marked that considerable differences can be observed using ordinary standardized mercury-in-glass thermometers calibrated to 0.2° F. The method adopted is admittedly crude, but the records obtained, Figs. 3 and 4, show that the climacteric period is characterized by a well-marked increase both in the temperature of the pulp and of the atmosphere of the respiration chamber. During senescence there is a gradual decline in pulp temperature, this being a variable feature according to the conditions of the experiment. From calorimetric measurements made by Langworthy and Milner (1912) it is known that the ripening of bananas is accompanied by the evolution of considerable amounts of heat, the rate of evolution being greatest at the commencement of ripening and diminishing during senescence. The differences between pulp and air temperatures within the calorimeter observed by these investigators were of the same order as those indicated in the present work.

## VIII. DISCUSSION: SOME ASPECTS OF RIPENING IN THE BANANA

(a) *The initiation of ripening.*

In the first paper in this series attention was directed principally to the papaw because it provided exceptionally favourable material for the study of ripening through its several regional phases. It was shown that the initiation of ripening in this fruit follows closely on the completion of seed develop-

ment, i.e. when the seeds have acquired their final thick indurated coat; this period was also noted as that in which the internal concentration of  $O_2$  reaches its maximal value. This evidence suggested that the completion of seed formation whereby a diversion of metabolites takes place, apparently acts as a stimulus in the initiation of ripening processes. The first trace of ripening is, in fact, to be observed in the seed stalks; this is followed by the appearance of ripening colour in the placentae and thence along the major vascular tracts through the pulp. In stone fruits such as the mango it has also been shown that the first tissues to show ripening changes are those abutting on the stone and that thereafter the progress of ripening is centrifugal (Wardlaw and Leonard, 1936 *b*).

In contrast to a seeded fruit such as the papaw the edible banana of commerce is quite seedless, the placentae being lined by abortive ovules which are of a brown colour from an early stage. The successive phases in ripening ascertainable by inspection in the papaw are therefore not so readily demonstrable in the banana: nevertheless, the first tissues to show ripening changes are those in the region of the placentae. Thereafter the progress of ripening at tropical temperature is outwards by way of the aggregates of vascular tissue in the region of the carpellary sutures. Finally, ripening changes are perceptible in the outer pulp and skin.

In the absence of normal seed development, what are the stimuli responsible for the onset of ripening?

In the first instance attention should be directed to the fact that ripening, as usually observed at tropical temperatures in the detached bunch or individual fruit, is in some respects in the nature of an artifact in that bunches harvested from the half-grown stage onwards will eventually ripen to a full yellow colour with sweet edible pulp and, in fact, apparently undergo some, though not necessarily all, of the changes associated with complete ripening. In that the detached bunch or finger consistently loses water which can no longer be replaced from the parent stem, changing water relations, known in other instances to promote the hydrolysis of carbohydrates, may play a part in the initiation of ripening.

It has also to be considered whether the onset of ripening in the banana can to some extent be elucidated by a closer analysis of the gaseous phenomena detailed above. It is known from the experiments of Kidd and West (1934) that the onset of ripening in apples is advanced by the application of high external concentrations of  $O_2$ . It has been observed that the rates of liberation of  $CO_2$  and water vapour show a rapid decline immediately after harvesting, which decline proceeds at gradually reduced rates until a new level is reached. It has also been observed that bananas held at variable temperatures ripen more rapidly than fruit held at a steady temperature, the mean temperatures in both being approximately the same. In that the internal gaseous phenomena in the two instances are by no means alike, attention is again directed to the part which these may play in the initiation of ripening.

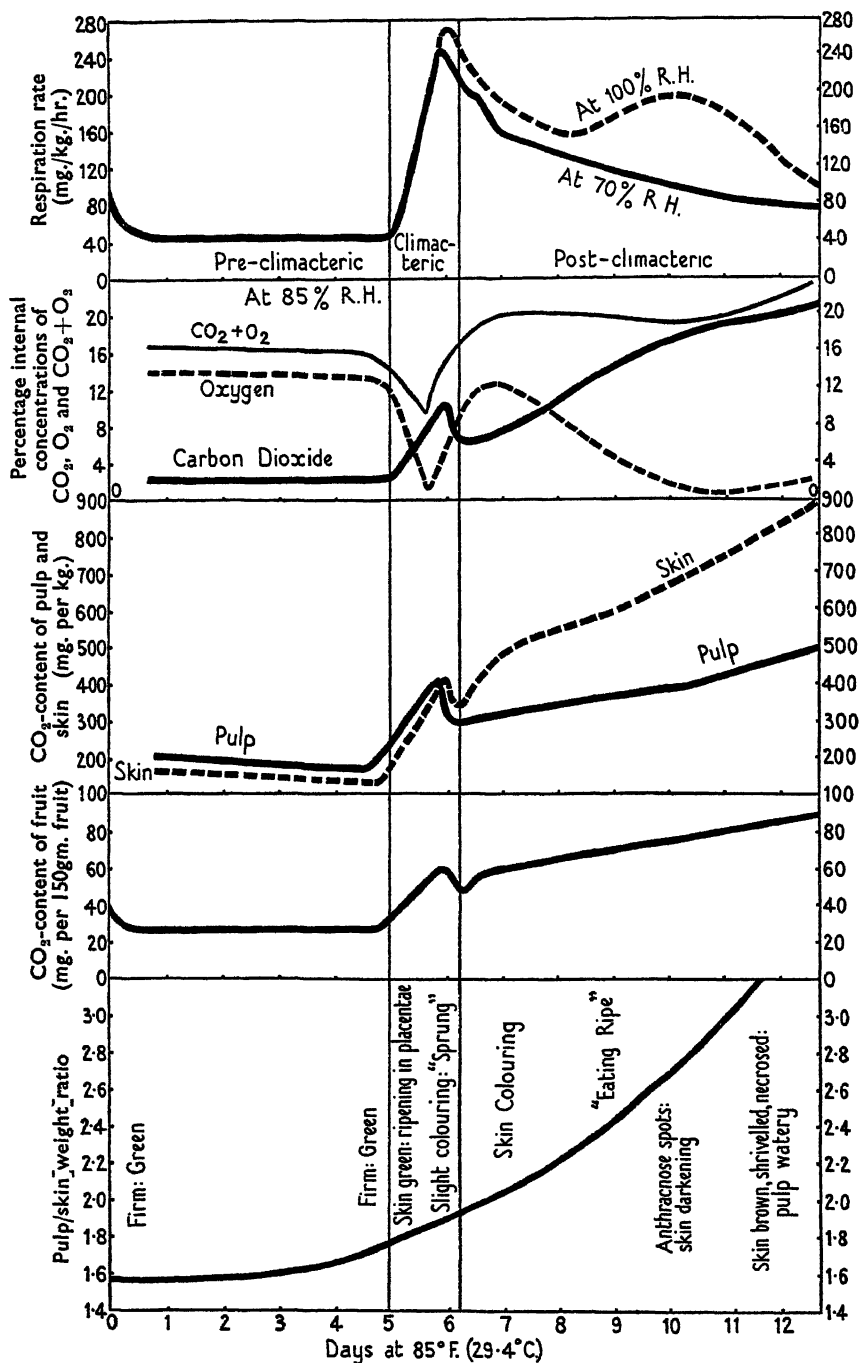


FIG. 23. Diagram showing the relationship in time between respiration rate, internal concentrations of  $\text{CO}_2$  and  $\text{O}_2$ ,  $\text{CO}_2$  content of pulp, of skin, and of whole fruit, also pulp/skin weight ratio, during the ripening of bananas at 85° F. (29.4° C.).

The influence of ethylene and other gases on the ripening of fruits has been the subject of much investigation. Regeimbal, Vacha, and Harvey (1927) observed that the effect of small doses of ethylene on the banana was to produce a temporary rise in the rate of liberation of  $\text{CO}_2$  followed by a decline to a value lower than normal. These workers consider that the results obtained 'may be due either to the increase of oxidation or to increase in the permeability of membranes allowing the diffusion of the  $\text{CO}_2$  already present in the cells'. Such observations would appear to support the theory of Nord and Francke (1928) that the effect of ethylene on ripening is due in part to temporary permeability changes within the cells.

A generalized view on the action of ethylene has been summarized by Gane (1936 *a*) as follows:

'Since bananas that have just started to ripen are only slightly affected by the addition of ethylene to the air, it may be concluded that the fruit itself is producing ethylene at this time. The addition of ethylene to the air will increase the concentration of ethylene inside the tissue; this may accelerate a process that has already started in all the cells, or it may raise the concentration above the threshold value in unchanged cells, and thereby cause these cells to ripen. The second alternative seems more feasible, since, when ripening once starts, it proceeds with the rapidity of a "trigger" action and an increased concentration above the threshold value is not likely to have any further effect.

'Ripening may be pictured as starting first in a few cells of a fruit. It is accompanied by the evolution of ethylene by these cells. The ethylene so formed diffuses into neighbouring cells, causing ripening to spread throughout the tissue.'

Important as the part played by ethylene in ripening may be, nevertheless a predisposition on the part of tissues to yield an immediate response on its application must be involved. The fundamental problem of ripening, on this analysis, is therefore seen to reside in a process or complex of processes other than the simple application of ethylene. While the data assembled here by the present writers on associated gas phenomena suggest that a partial explanation of the initiation of ripening may be forthcoming from further investigation along these lines, the information at present available is insufficient and too indirect to permit of any definite hypothesis.

Further aspects of permeability changes in relation to the initiation of ripening have been suggested. In the tomato Gustafson (1929) has suggested that the climacteric rise is due to a lowering of the H-ion concentration of the cell sap; Kidd and West (1935) and Kidd (1935), discussing the climacteric in apples, also envisage the possibility of a decrease in acidity to a critical point whereby the permeability of the cytoplasm to fructose is increased, making possible an increase in respiration rate. For the banana, however, Gane (1935, 1936 *b*) has recorded an increase in acidity, from a pH of 5.8 to 4.3, during the climacteric period. Barnell (1940 *b*) has observed that the titratable acidity of banana pulp consistently rises during the climacteric phase. While changes in protoplasmic permeability and, by consequence, in

respiration rate may be related to acidity changes, the evidence available is clearly not sufficiently complete or consistent to permit of any definite generalization being made at this stage.

The general theory of a change in permeability, whereby enzymes gain access more freely to their respective substrates, has been frequently invoked in explanation of the increase in respiration rate that accompanies the onset of ripening. In a general discussion of the Pasteur effect, Dixon (1937) has suggested that 'the expenditure of energy by [oxidative] respiration is responsible for maintaining part of the cell enzymes inaccessible to the substrate'. In anaerobiosis, on the other hand, it is considered that the cell becomes more permeable, so that the rate of carbohydrate catabolism is greater than in the presence of oxygen. It may be assumed that there is a threshold value of oxygen concentration at which changes in permeability begin to be operative, these effects increasing in intensity with further decreases in oxygen concentration. Without elaborating the many implications involved in permeability theory as specifically related to oxygen, it will be seen that the data presented in this paper on the downward trend of the internal concentration of  $O_2$  in the banana during the pre-climacteric phase are such as to indicate the probable importance of oxygen as a factor involved in the initiation of ripening.

*(b) The progress of ripening as related to the organography of the fruit.*

Although the respiration curves submitted here for the banana are of a considerably more complex nature than those previously obtained for the papaw, nevertheless the progress of ripening is in most essential respects marked by analogous changes. Fig. 23, which is diagrammatic (but based on data set out in the foregoing sections), shows the correlation between the major ripening changes observed in the banana and the respirational changes measured.

Immediately after the harvesting of the bunch and the removal of the experimental finger, the initial respiration rate is high, but falls rapidly. It would thus appear that the detachment of a fruit considerably modifies its physiological behaviour. This point of view can be extended to detached hands and to the bunch itself. Transpiration measurements (Leonard, 1940) also support this view. Similar changes immediately after harvesting have been recorded by Benoy (1929) for a number of fruits and vegetables.

The pre-climacteric phase is characterized by high turgidity, by green to pale green skin colour (according to the environment during growth), and by a pulp which is crisp to the centre. Relevant anatomical data will be given in a later paper in this series. In the several curves presented in Fig. 23 it will be noted that only slight trends are exhibited during this phase: nevertheless, the metabolic changes taking place are those which prepare the way for the very conspicuous changes associated with the climacteric. Accordingly the

writers consider that more detailed investigation of this phase will yield important data on the initiation of ripening.

The first indication of ripening is to be observed, during the early stages of the climacteric, in the placental region. There, in a clearly defined zone, the tissues become soft, acquire a honey colour, and are sweetish to the taste. At this stage the outer pulp tissues are still crisp and full of starch. This is the stage at which the internal concentration of oxygen at the centre of the fruit falls rapidly to a low value while the curve of respiration rate rises towards its peak.

The initiation of ripening in the placental region is quickly followed by ripening in the outer pulp, the outstanding feature being the rapid disappearance of starch. At this stage the skin, as a rule, is still firm, green, and turgid, but the pulp is seen to have undergone some softening. It is during this brief phase that the internal concentration of  $O_2$ , having reached its minimum value, shows a marked recovery to higher values while the  $CO_2$  peak values, both in respiration rate and in internal concentration, are reached.

The downward trend in the internal concentration of  $O_2$ , and the rapid attainment of minimal values which are conspicuous features of the climacteric phase, thus afford an indication of increased metabolic changes in the placental region—an inference supported by the rise in pulp temperature recorded at this time, as well as by increased respiration rate, and increased internal concentration and content of  $CO_2$ .

The diminution of internal  $O_2$  concentration may have important physiological effects. By analogy with numerous experiments in which the respiration of fruit has been estimated first in air and then in an atmosphere of  $N_2$  or  $H_2$ , the high rate of liberation of  $CO_2$  over a brief period in such atmospheres has been regarded as being in the nature of a 'transition effect' (Gustafson, 1930; Smith, 1934; Wardlaw, 1936). In experiments with the banana Gane (1936 *b*), however, found that on transferring fruits on the climacteric rise to an atmosphere of pure  $N_2$ , there was a rapid decline in respiration rate, but in so far as his observations were made at intervals of twenty-four hours, rapid transition effects, i.e. an increased respiration rate followed by a decreased rate, could have occurred without being recorded. It should also be noted that his observations were made at  $15^\circ C.$  ( $59^\circ F.$ ).

In respiration studies with Bartlett pears, Magness and Ballard (1926) found that during ripening there was an increase in the internal concentration of  $CO_2$  and a decrease in the  $O_2$  concentration, but it would appear that they did not arrive at any clear understanding of the several changes taking place.

On analysis, the rise to the climacteric peak may be attributed to (i) increased  $CO_2$  production firstly by metabolic changes in the placental region and subsequently in the outer pulp, and (ii)  $CO_2$  liberated from the tissues as a transitional effect, causally related to (a) low  $O_2$  concentrations and (b) the



increase in the temperature of the pulp. The regional differences in  $\text{CO}_2$  concentrations in placentae and outer pulp, described in section IV, contribute to an explanation of the form of the respiration rate curve about the climacteric peak, i.e. it represents the summation of different and interacting interrelated phases in ripening.

In those instances where the shape of the respiration rate curve is considered to have resulted, in part, from a physical transition effect calculations of the amount of heat liberated at that time based on  $\text{CO}_2$  output alone may be subject to error.<sup>1</sup>

In commenting on the transition effect of a rise in temperature upon the rate of respiration Blackman and Parija (1928) note:

'One considerable difficulty that stands between the observer and elucidation of these transitional phenomena is that the intensity and duration of an intracellular production of  $\text{CO}_2$  is so much distorted when the observed signs of it are only the intensity of duration of outward escape of  $\text{CO}_2$  by diffusion through the surface of the massive tissues of an apple. Should a few cubic centimetres of  $\text{CO}_2$  be suddenly produced in excess by the respiratory mechanism, which before and after maintained a steady state, then this would not manifest itself outside as a sudden output of  $\text{CO}_2$  of identical timing and intensity, but as an output which might rise fairly quickly to a maximum but would decline quite slowly again towards the steady rate, owing to diffusive lag. The decline would take the form of a 'logarithmic curve'. It is clear that when we observe the  $\text{CO}_2$  production of an apple falling in a curve of this sort from a high level, and taking many hours to complete the falling transition, we cannot conclude that any actual internal production of  $\text{CO}_2$  has been continuing at a heightened rate beyond the point of time at which the high level ceased and the fall began. The observed escape of  $\text{CO}_2$  may be described as a distorted anamorph of the production.'

This may well be extended to all transition effects in studies of respiration in which the only measurement made is that of liberated  $\text{CO}_2$ .

A further factor which may be operative in yielding the high respiration rates observed at the climacteric has been suggested by Willaman and Brown (1930), namely, that the presence of high concentrations of  $\text{CO}_2$  in the tissues may cause an actual increase in rate of production of  $\text{CO}_2$ , in consequence of the effect which the increased acidity of the sap may exercise on protoplasmic permeability. As a result of experiments in which fruits and vegetables were stored in atmospheres containing high  $\text{CO}_2$  and also oxygen, Thornton (1933, *a* and *b*) showed that there was an actual decrease in acidity, though his interpretation of the data obtained is open to criticism in view of his neglect of a consideration of other changes in metabolism.

The rapid recovery in  $\text{O}_2$  concentration in the placental region during the climacteric may be regarded as indicating the termination, in that region, of the period of rapid oxidative metabolism. It is also an indication that, up

<sup>1</sup> For bananas, Langworthy and Milner (1912) have estimated that 2.58 calories of heat are produced for each gram of  $\text{CO}_2$  liberated.

to this stage in ripening, the outer tissues (pulp and skin) still retain more or less unchanged their original low resistance to the movement of gases. As a rule the first colour changes in the skin are not apparent until the peak of respiration rate is reached or passed.

As the respiration rate curve descends from the climacteric peak value the fruit attains to what has been described as the 'sprung' condition; at this stage the skin has become yellowish-green and, although still somewhat crisp and elastic, has definitely lost some of its original turgidity, while the softening of the pulp within permits it to yield under gentle pressure.

The subsequent ripening of the outer pulp and skin through the 'eating-ripe' to the final senescent phase is marked by a second but slower reduction in the internal concentration of oxygen. Coincident with this, the concentration of  $\text{CO}_2$  rises, but without producing a commensurate increase in the respiration rate. It is during this period, which is accompanied by the full development of skin coloration, that tissues, as indicated by decreasing pressure in attached manometers and by the diminished sum value of  $\text{CO}_2$  and  $\text{O}_2$ , begin to exercise an increasing resistance to the movement of gases.

In some preliminary experiments on the influence of different external atmospheres on the ripening of Cavendish bananas Kidd and West (1932) showed that during the *post-climacteric* period the response in respiration rate to oxygen concentrations varying from 5 to 21 per cent. at  $18^\circ \text{C}$ . ( $64.4^\circ \text{F}$ .) did not show any marked variation, though the time required for ripening was decreased at the higher  $\text{O}_2$  concentrations. The response to carbon dioxide atmospheres varying from 5 to 30 per cent., gauged by the number of days taken to ripen by comparable lots of fruit, was very uniform. That normal ripening was obtained in  $\text{O}_2$  concentrations from 5 per cent. upwards is not surprising in view of the data now available on the actual concentrations present within the fruit during the ripening process. Further, these results are what might be expected if tissue resistance to the movement of gases was a factor of major importance as indicated by the current studies.

In this connexion reference may be made to Olney's (1926) values for the respiratory quotient ( $\text{CO}_2/\text{O}_2$ ) for the banana which show an increase from 1.01 to 1.24 during ripening and senescence. In recent experiments with Bramley's Seedling apples at  $22.5^\circ \text{C}$ ., Kidd and West (1938) have shown that the uptake of  $\text{O}_2$  follows the general trend of the respiration rate curve. The respiratory quotient was 1.02 during the pre-climacteric stage, i.e. immediately after harvesting; it rose to 1.25 at the peak of the climacteric, and thereafter more slowly to 1.40 in twenty-four days. By applying pure  $\text{O}_2$  to a post-climacteric fruit  $\text{CO}_2$  production and uptake of  $\text{O}_2$  were increased, with the result that the respiratory quotient fell from 1.42 to 1.20. Such results for the apple are in harmony with the views put forward by the present writers on the relationship between access of  $\text{O}_2$  to the tissues, increase in the  $\text{CO}_2$  content of the tissues, and the increase in resistance to gaseous movement during senescence.

During the ripening of tomatoes, Singh and Mathur (1936, *a* and *b*) have shown that increasing amounts of  $\text{CO}_2$  accumulate in the tissues as has now been demonstrated for the banana, Fig. 23 and section VI. They conclude that, in massive structures like ripe tomatoes where the tissues offer resistance to the movement of gases, 'the total  $\text{CO}_2$  production during metabolism is not the same as that evolved at the surface'. And whereas these investigators were able to demonstrate an increasing tissue content of  $\text{CO}_2$  during ripening, the amount of gas that could be extracted from the tissue, using a Torricellian vacuum, declined after the climacteric peak had been passed, indicating increased resistance to the movement of gases. This was also borne out by the decrease in the respiration rate during senescence.

The stage at which bananas become eating ripe is definitely post-climacteric. This is in keeping with Gane's (1936 *b*) observations on fruit which had been previously subjected to cold storage, and differs from the finding of Kidd and West (1936) in respect of pears where the stage of optimum eating quality coincides with the peak of respiration activity.

It is at a certain and usually well-defined stage in the senescence of banana fruits that the first development of latent infections becomes apparent, this being followed almost immediately, or soon after, by a typical spreading brown necrosis of the skin, and softening to the point of liquefaction of the pulp. Thereafter the skin may be overrun and exploited by various secondary fungi. Nevertheless, in contrast to previous published curves (cf. Kidd and West 1930, Kidd 1932, and Gane 1936, *a* and *b*), it has not been the present writers' experience with the banana that either the initial appearance of fungal wastage, or the later stages, are marked by a rapid increase in the liberation of  $\text{CO}_2$ .

The close similarity between manometer records of pneumatic pressure and the internal gas concentrations associated with the several changes described above has been demonstrated and discussed in section V. The recovery of the manometer from high negative values to approximately zero value during the ultimate phase of senescence is closely associated with a well-marked rise in the internal concentration of  $\text{CO}_2$ , i.e. the  $\text{CO}_2$  previously retained in the cells is being liberated.

Again, during the period of final senescence it has been found that after the internal concentration of  $\text{O}_2$  has fallen to a low value, e.g. 1–2 per cent., there may follow a period during which this concentration slowly rises, the internal concentration and tissue content of  $\text{CO}_2$  also rising. This increase in  $\text{O}_2$  concentration may be taken as an indication that  $\text{O}_2$  is no longer being actively utilized. It is during this period that fruits become noticeably aromatic, with a suggestion of alcohol accumulation in the tissues. In this connexion the work of Thomas (1925) and others is of interest. Thomas has shown that when apples are maintained in certain concentrations of  $\text{CO}_2$ , even in the presence of abundant  $\text{O}_2$ , the oxidative system is inactivated, whereas the zymase system remains unaffected. By analogy it may be argued

that comparable conditions obtain in the tissues of bananas during *final* senescence—this period being characterized by increasing acidity, continued  $\text{CO}_2$  production, failure to utilize  $\text{O}_2$ , and the formation of alcohol and other odorous compounds. In a later paper, Thomas (1931) discusses critically the part which increased tissue resistance to gaseous movement may play in causing zymasis: he concludes that this does not afford a universal interpretation of the available data from studies of apples and pears and suggests that 'during the disorganization of pears and apples, the intricate co-ordination of enzymes in the respiratory centres of the protoplasm breaks down; then, so long as the zymase component remains active, carbohydrate cleavage proceeds in part, at least, all the way to ethyl alcohol and acetaldehyde'.

In a study of respiration and transpiration in ripe Conference pears Luthra (1924) observed that fruits held in a dry atmosphere both respired and transpired at a higher rate than in moist air. Somewhat similar results are recorded by Gerhart (1930) for strawberries. Such records of higher rates of respiration in dry air during senescence are not in keeping with the results obtained for bananas during the present study. In pears the greater loss in weight in dry air was also accompanied by wilting and earlier breakdown.

In conjunction with the present work on respiration, biochemical studies on bulk samples have been undertaken (Barnell, 1940*b*). In addition, biochemical investigations have also been carried out during the ripening of a series of fruits whose precise stage of maturity at the time of sampling was known from respiration studies. An exact correlation of respiration and biochemical change has thus been rendered possible. The relevant data are now available and will be made the subject of a later communication in this series. No further discussion of this aspect will therefore be undertaken at this point.

## IX. SUMMARY

1. The respiration during ripening at  $85^\circ\text{F}$ . ( $29.4^\circ\text{C}$ .) of individual banana fruits of standard grades was investigated by methods yielding data on respiration rates at different relative humidities, on internal concentrations of  $\text{CO}_2$  and  $\text{O}_2$ , on  $\text{CO}_2$  content of tissue, on variations in pneumatic pressure, and on changes in pulp temperature.

2. The respiration rate indicates that important and well-marked changes take place during ripening. In the freshly harvested, turgid green fruit, the respiration proceeds initially at an approximately steady rate. With the onset of ripening this is succeeded by a rapid rise to the climacteric peak value. This is followed by a decline during which time the fruit shows colouring of the skin, and reaches what has been described as the 'sprung' or softening condition. Subsequently, as the fruit matures to the 'eating ripe' and 'boiled ripe' stages, the respiration rate proceeds at a new and approximately steady, or slightly rising, high rate. The final senescent phase when the pulp becomes watery and the skin dark, necrosed, and subject to fungal attack is characterized by a gradual diminution of respiration rate.

3. During the pre-climacteric and climacteric phases a close and direct relationship, both quantitative and in time, exists between the internal concentration of  $\text{CO}_2$  and the respiration rate, but as further ripening changes take place in the tissues, this relationship no longer obtains.

4. During the pre-climacteric phase the internal concentration of  $\text{O}_2$  is relatively high but shows a slight decline with time. The period of the climacteric rise in respiration rate is one of active utilization of  $\text{O}_2$  and is characterized by a rapid decline in its internal concentration; in some instances this may reach almost the extinction point. In respect of time, the point of minimal internal concentration of  $\text{O}_2$  slightly precedes the attainment of the climacteric peak values shown in the respiration rate and internal concentration of  $\text{CO}_2$ . By analogy with certain other experiments involving the effect of low concentrations of  $\text{O}_2$  on respiring tissues, it is suggested that, to some extent, the high respiration rates observed in the rise to the climacteric peak are in the nature of a transition effect. Subsequently as ripening proceeds through the climacteric peak to the 'sprung' condition there is a rapid recovery in the internal concentration of  $\text{O}_2$  followed by a further decline as the fruit approaches the 'eating ripe' condition.

5. The general factors involved in the relation of transpiration to respiration are considered in detail. In experiments which are described the influence of transpiration on respiration is shown to be important, the shape of the respiration rate curves and of the curves of internal concentrations of  $\text{CO}_2$  and  $\text{O}_2$  obtained from fruits held at low humidities being affected in a manner which is explicable in terms of the increased resistance to the movement of gases offered by the tissues in consequence of their partial desiccation.

6. Ripening in the banana is regional, proceeding from the central placental region through the outer pulp to the skin. The distal, i.e. stylar, end ripens slightly in advance of the proximal or stem end. These conclusions, which have been ascertained from regional analyses of the internal gas concentrations during ripening as well as by inspection, are in agreement with the facts of regional ripening observed in other fruits.

7. The trends of changing pneumatic pressure within fruits during ripening as observed by manometers support the data on the changing internal concentration of  $\text{CO}_2$  and  $\text{O}_2$ . Pneumatic pressure is also appreciably altered by the rate of transpiration, high transpiration rates producing an increase in the negative pressure within fruits.

8. An investigation of the  $\text{CO}_2$  content of fruits has shown that there is a direct relationship, though not necessarily a proportional one at all stages, between the  $\text{CO}_2$  content and the internal (i.e. air-space) concentration of  $\text{CO}_2$  during the several phases of ripening. But whereas in the pre-climacteric phase the  $\text{CO}_2$  content is higher in the pulp than in the skin, the post-climacteric phase is characterized by the reverse relationship.

9. Observations of fruit temperatures during ripening show that: (a) at steady external air temperature the temperature of the pulp is closely related

to the transpiration rate, which again is influenced by the humidity and rate of movement of the air, (b) the climacteric phase is characterized by a marked rise in temperature.

10. The collective data presented show that during ripening and senescence a succession of well-defined phases can be observed: (a) the pre-climacteric, firm, green stage characterized by a low internal concentration and tissue content of  $\text{CO}_2$ , low respiration rate and relatively high but decreasing internal concentration of  $\text{O}_2$ . (b) The climacteric phase, in which the curve of respiration rate rises steeply to a peak value, is characterized by a rise in pulp temperature, by a sharp decline, almost to the extinction point, in the internal concentration of  $\text{O}_2$ , and by a corresponding rise in the internal content and concentration of  $\text{CO}_2$ , the latter reaching a peak value which coincides in time with the peak of respiration rate. (c) The post-climacteric peak phase shows a decline in respiration rate and in the internal concentration and content of  $\text{CO}_2$ , and a well-marked recovery in the internal concentration of  $\text{O}_2$ ; at this stage skin colouring and the softening condition described as 'sprung' may usually be observed. (d) In the next phase, up to the point at which the fruit becomes 'eating ripe' there is a steady or slightly increasing respiration rate, a rising internal concentration and content of  $\text{CO}_2$  and a decline in the internal concentration of  $\text{O}_2$ ; during this phase it is evident that there is increased resistance to the movement of gases. (e) During the over-ripe and final senescent phase, the pulp becomes watery and the skin brown, necrosed, and subject to fungal rotting, and increased resistance to the movement of gases becomes an increasingly conspicuous feature; there is a steady or slightly declining respiration rate and a rise in the internal concentration and content of  $\text{CO}_2$ ; the trend of the internal concentration of  $\text{O}_2$  continues towards the extinction point, but may show a slight increase later, the indication being that it is no longer being utilized in metabolism.

From the foregoing observations it will be seen that the essential feature of the phase of ripening known as the climacteric, though exhibiting considerable variation under different experimental conditions and with different categories of fruit, is a change from a lower to a higher level of metabolic activity.

11. A discussion is presented of the factors which may be involved in the initiation of ripening; it is shown that the part played by gases, in particular by  $\text{O}_2$ , merits careful consideration. Ripening as an expression of organography is also discussed, data being given which show that the results obtained conform to what has been observed in other fruits, namely, that ripening is initiated in the central placental region, which in the banana contains the abortive ovules, and proceeds outwards to the skin.

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# Respiration and Maturity in Peaches and Plums

BY

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With six Figures in the Text

THE respiration of detached fruits or other senescent plant organs, such as leaves, kept in the dark at a constant temperature, usually shows a number of distinct phases. There is commonly a fall in the respiration rate to a low level followed by a rise to a peak. This rise is associated with the ripening of the fruit, or the yellowing of the leaf, and has been called the climacteric (Kidd, 1935). After the climacteric peak is passed, respiration tends to decline steadily until the tissues die or suffer invasion by saprophytic organisms.

The phenomenon of the climacteric, or 'senescent rise in respiration', has been described for leaves (Godwin and Bishop, 1927) and for such fruits as the apple (Kidd, loc. cit.), pear (Kidd and West, 1936), tomato (Singh and Mathur, 1936), banana (Crane, 1936), and papaw (Wardlaw and Leonard, 1936). Most of the published accounts deal with mature or nearly mature fruits, and little information is available regarding the behaviour of young fruits during continued storage. Some workers (Kidd, Wardlaw, and Leonard) give curves showing changes in the intensity of respiration during growth and maturation (inferred from the behaviour of fruits immediately after picking), but they do not state whether young fruits show a climacteric or not. There is a general idea that such fruits fail to ripen normally.

Godwin (1927) has shown that young leaves of the cherry laurel exhibit no climacteric and do not yellow. But 'with increasing age from unfolding a corresponding increase in the size of the "respiration hump" is found', accompanied by an increasing tendency to yellow.

In the present paper the results of an investigation of the respiration of peaches and plums are described. They indicate a complex relationship between maturity, as revealed by respiratory behaviour during storage, and the chronological age of the fruit during growth. Though considerable differences between peaches and plums were revealed in intensity of respiration, length of life, and other characteristics, the nature of this relationship seems to be fundamentally the same in the two fruits investigated.

## MATERIALS AND METHODS

The fruits used were the Peregrine peach and the Kelsey plum, the latter a variety of *Prunus salicina*. They came from trees on a farm in the Cape

Province some thirty miles from the laboratory. Respiration records began four to six hours after the fruit was picked. The fruits used in the respiration experiments were taken from larger samples picked by a method of random selection. Fruits of size- and colour-average for the given sample were chosen. In the case of fully grown peaches and plums single fruits were generally used; but larger samples were taken in the case of small, immature fruits. They were placed in light-proof containers, which were immersed in a water-bath kept at a temperature of 25° C. By means of a Dinton air-pump an air stream, freed from CO<sub>2</sub>, was passed over the fruit at a speed of 3–4 litres an hour. The air entering the chambers was not quite saturated and the fruit lost a certain amount of weight during the course of the experiments. In the longest experiment (plums kept for over two months) the fruit lost 8 per cent. of its original fresh weight. In most cases the loss did not exceed 3–4 per cent.

The CO<sub>2</sub> evolved was observed by baryta in Pettenkofer tubes. A 'gas commutator', consisting of a series of mercury valves, activated by a clock and falling weight, and made largely of Meccano parts,<sup>1</sup> enabled readings to be taken at any desired intervals. Though three-hour readings were usually taken, the results, expressed in ml. CO<sub>2</sub> per hour per kg. original fresh weight, were plotted as six- or twelve-hour averages and gave fairly smooth curves.

A difficulty was encountered in the case of very ripe fruit which tended to go mouldy, sometimes before the climacteric peak was reached. The records also suffered from the fact that, in the case of plums, the fruit was in some cases so long-lived that quite healthy samples had to be discarded to make way for samples picked at a later stage: the gas commutator did not allow of more than eight respiration experiments being conducted simultaneously.

Measurements of respiration formed part of a more comprehensive investigation involving chemical analysis of samples of fruit immediately after picking and at intervals during storage at 25° C. Only the respiration data are included in the present paper.

## RESULTS

### *Peaches.*

Peaches were picked at approximately weekly intervals from December 13, 1938. On this date, six weeks from petal-fall, the fruits were small, green, and hard. The seventh and final sample was picked on January 21, when the fruit had attained its full size and had coloured (red on green background), but was still firm. Details of the appearance of the fruit before and after storage are given in Table I. Respiration curves are given in Figs. 1 and 2. Fig. 1 is in chronological form. The thick black line joining the points representing CO<sub>2</sub> output immediately after picking gives the assumed respiration drift of the fruit on the tree. The thinner lines show the respiration of the different samples during storage at 25° C. In Fig. 2 the respiration curves are plotted

<sup>1</sup> To be described elsewhere.

TABLE I. *Peaches.*

Sample.	Date of picking.	Number of fruits.	Weight per fruit (gm.).	Condition when picked.	Days to beginning of climacteric.	Days to climacteric peak.	Days to end of Experiment	Condition at end of experiment.
A	Dec. 13	6	25.1	firm and green	1.5	8	15	firm, yellow, and red (2 fruits slightly mouldy)
B	Dec. 20	4	33.1	firm and green	1	6	14	firm, greenish-yellow and red
C	Dec. 28	3	41.4	firm and green	4	7	14	firm and yellowish-green (slight internal browning)
D	Jan. 4	3	55.1	firm and green	18	30	30	firm, yellow, and red (1 fruit slightly mouldy)
E	Jan. 11	2	94.0	firm and green	4	—	12	(both fruits very mouldy)
F	Jan. 19	1	128.0	firm and green	1	—	4	(fruit very mouldy)
G	Jan. 21	1	128.0	firm, green and red	0	5	10	soft, yellow, and red (skin splitting)

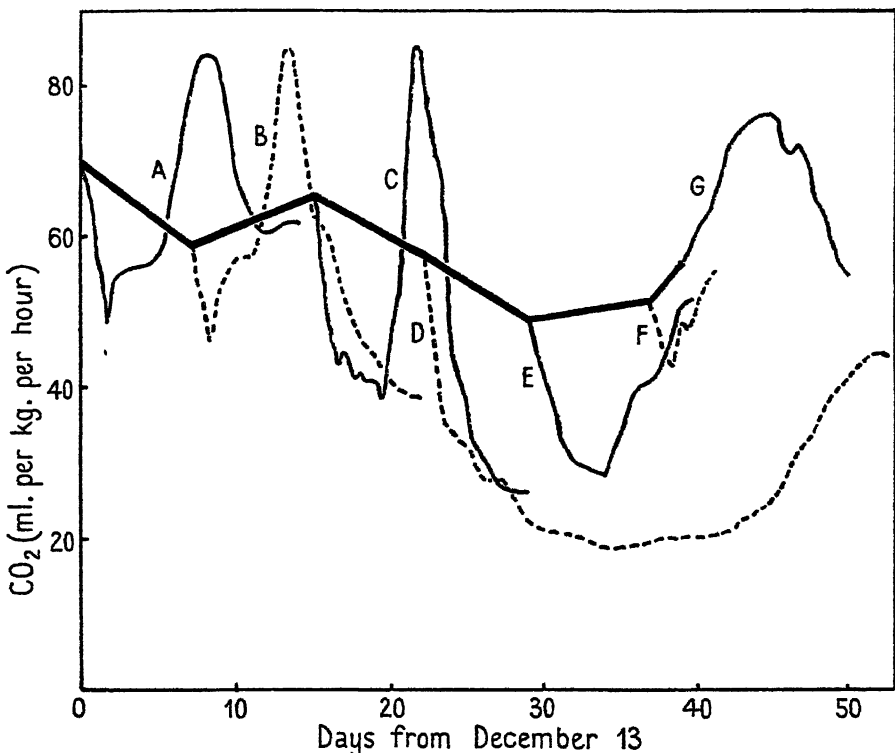


FIG. 1. Respiration of successive samples of peaches (Peregrine) picked at intervals during growth.

from zero on a time axis representing days in store. Successive samples are denoted by the letters A, B, C, &c., and are the same in Figs. 1 and 2.

A, B, and C gave curves very similar in form. All had an early and pronounced climacteric. In the interval of seven days between the picking of

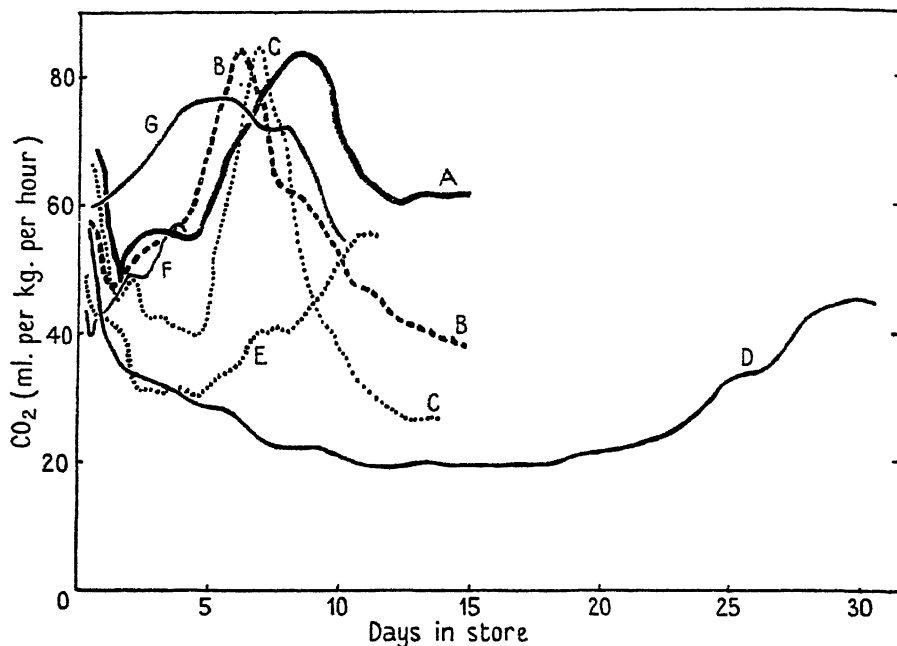


FIG. 2. Respiration of successive samples (A-G) of peaches (Peregrine) picked at intervals during growth. For dates of picking see Fig. 1.

C and D the fruit on the tree appeared to undergo a profound change. The respiration of D declined steadily for twelve days to a low level and then remained steady for about a week. It then rose slowly to its climacteric, reaching a peak thirty days after picking, at a level much lower than that shown by the previous samples. Thus D was longer lived and much less 'mature' on picking than A, B, or C. The interval between picking and the beginning of the climacteric rise was 1.5 days, 1 day, 4 days, and 18 days for A, B, C, and D respectively. The corresponding intervals between picking and the climacteric peak were 8, 6, 7, and 30 days. Thus there was a tendency for the pre-climacteric interval to shorten, after which it lengthened considerably.

Judged by the development of colour, A, B, C, and D gave a straightforward sequence, showing that the fruit on the tree was becoming progressively less mature, in the sense that it required a longer period to ripen in store. After fourteen days from picking A had lost all its chlorophyll and had developed an appreciable amount of red epidermal pigment (red on yellow background); B after a similar interval in store had a certain amount of red colour, but the

chlorophyll had only partly disappeared (red on greenish-yellow background); C after fourteen days had begun to yellow but had not developed any red pigment at all (yellowish-green); D, fourteen days after picking, was still in the pre-climacteric stage and was completely green—it finally assumed the colour of a fully ripe peach (red on yellow background) after thirty days in store.

None of the samples A–D showed any softening of the tissues even in the post-climacteric stage.

From this point onwards each successive sample ripened more quickly than the previous one. In other words, in the sequence A–D increase in chronological age coincided in general with a retrogression in physiological maturity, but in the sequence D–G there was an advance in physiological maturity with increasing age. In E the respiration rate fell for about four days and then passed into a climacteric rise without any appreciable intervening level period. The climacteric rise was more pronounced than in D. F began its climacteric rise very shortly after it was picked, and G was already in the climacteric stage when removed from the tree. The development of moulds in E and F made it impossible to determine the position of the climacteric peak or the colour of the skin and the condition of the flesh at the end of these two experiments. In G the red colour had already appeared prior to picking; the chlorophyll disappeared after a few days in store and the tissues became soft.

### *Plums.*

Samples of plums were picked at intervals between December 13 and February 27. At the latter date the fruit had reached the commercial picking stage. Details are given in Table II. The plum was found to differ markedly from the peach in its slower rate of growth and development on the tree, its lower rate of respiration (the rate of  $\text{CO}_2$  output was about half that of the peach), and its longer life in store.

Respiration curves for the plum are given in Figs. 3 and 4. Sample A showed a definite climacteric rise after eleven days, though the rate at the climacteric peak was far short of the initial rate. B had an earlier but less pronounced climacteric. These rises in respiration were accompanied by only very slight yellowing; no red colour was developed.

Samples C and D were discarded while still green and before they had given any indication of a rise in respiration. E began to yellow very slowly after about thirty days, but there was no sign of the climacteric until about two months after picking, when a slight rise in  $\text{CO}_2$  output was noted. This coincided with the first appearance of red colour. E had a longer life in store than any of the samples picked subsequently. From the appearance of the curves (Fig. 3) it seems probable that D, and possibly C also, would have been even longer-lived than E had their respiration been followed. In the plum the pre-climacteric level phase was much more obvious than in the peach. The level of respiration during this period seemed to be inversely correlated with

TABLE II. *Plums*

Sample.	Date of picking.	Number of fruits.	Weight per fruit (gm.).	Condition when picked.	Days to beginning of climacteric.	Days to climacteric peak.	Days to end of experiment.	Condition at end of experiment.
A	Dec. 13	6	23.7	firm and green	11	14	22	firm and yellowish-green
B	Dec. 28	3	43.1	firm and green	2	3	22	firm and yellowish-green
C	Jan. 11	2	68.7	firm and green	—	—	19	firm and green
D	Jan. 25	4	92.5	firm and green	—	—	6	firm and green
E	Feb. 2	1	101.0	firm and green	61	—	68	firm, yellow, with traces of red (mould growth beginning)
F	Feb. 10	1	116.4	firm, and yellowish-green	19	23	26	firm and greenish-yellow
G	Feb. 18	4	137.0	firm, yellowish-green, with traces of red	12	16	19	soft, yellow, and red,
H	Feb. 27	3	138.0	firm, yellow, and red	0	5	7	soft, yellow, and red

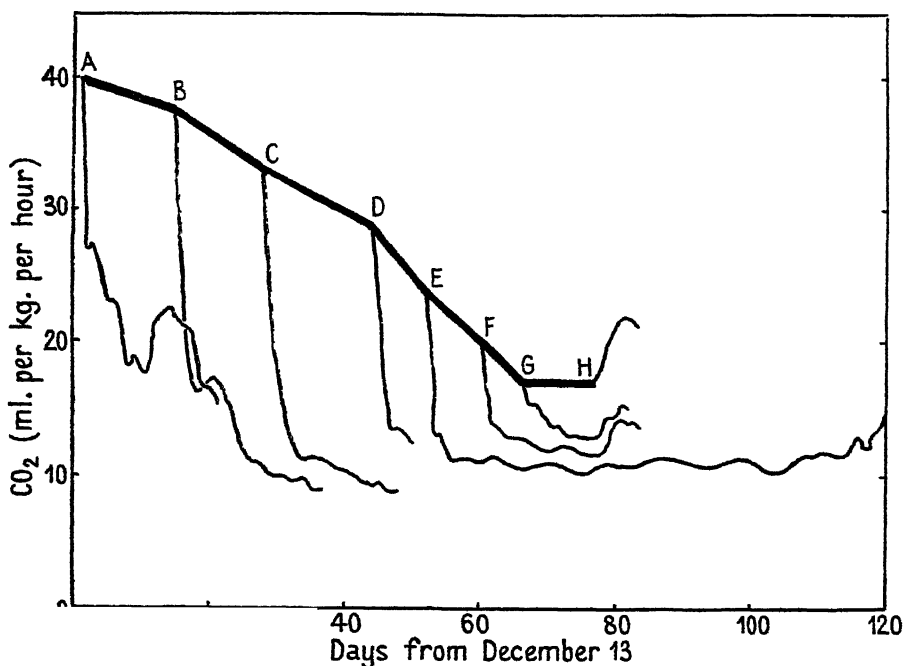


FIG. 3. Respiration of successive samples of plums (Kelsey) picked at intervals during growth.

length of life, the lower the level the longer the interval before the onset of the climacteric.

Samples F, G, and H were progressively riper on picking. Sample H gave a climacteric rise from the start.

In general the plums seemed to pass through the same series of stages as the peaches.

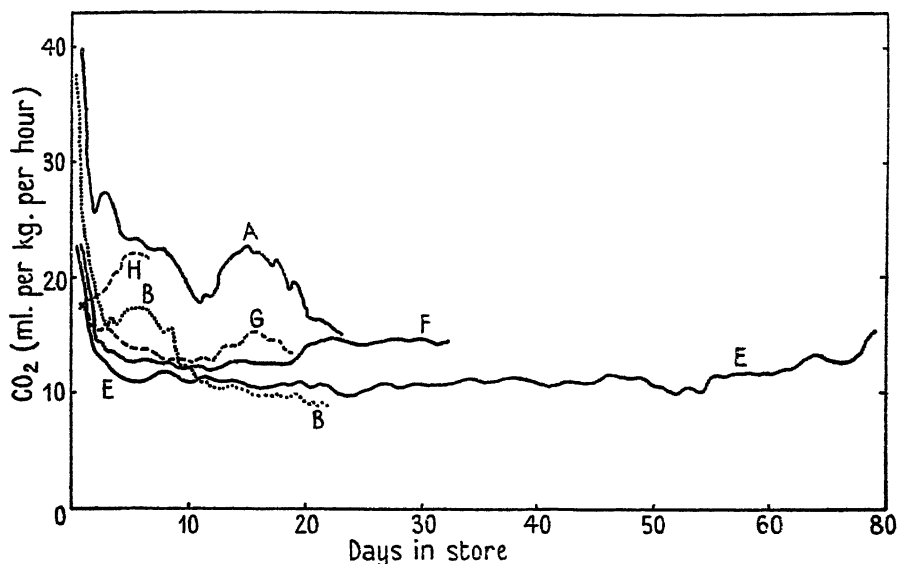


FIG. 4. Respiration of successive samples (A-H) of plums (Kelsey) picked at intervals during growth. For dates of picking see Fig. 3.

## DISCUSSION

In the life of both the peach and the plum, as revealed by the course of respiration after picking, there appear to be three distinct phases. Young fruits have an early climacteric. In fruits of intermediate age the climacteric is much delayed. Older, nearly fully grown, fruits again show an early climacteric, and in fully developed fruits the climacteric may already have begun on the tree. An attempt has been made in Figs. 5 and 6 to represent graphically the relation between physiological 'maturity' and chronological age. The intervals between picking and the beginning of the climacteric rise are plotted in sequence according to date of picking. Both for the peach and the plum they give a regular curve (*w-x-y-z*) which falls slightly, then rises steeply, then falls again. In the plum, part of the curve—at the period of maximum longevity—is missing, owing to the fact that samples C and D were discarded before the climacteric was reached.

If proximity to the climacteric is taken as an index of maturity in pre-climacteric fruit, it is possible to state that young fruits are more 'mature' than



fruits of intermediate age. The 'ripening' of young fruits is not ripening in the commercial sense, since it does not result in the fruit becoming edible, but it is accompanied by some of the phenomena associated with normal ripening, viz. climacteric rise in respiration and (more markedly in the peach than in the plum) the development of colour. Fruits of intermediate age are less 'mature'

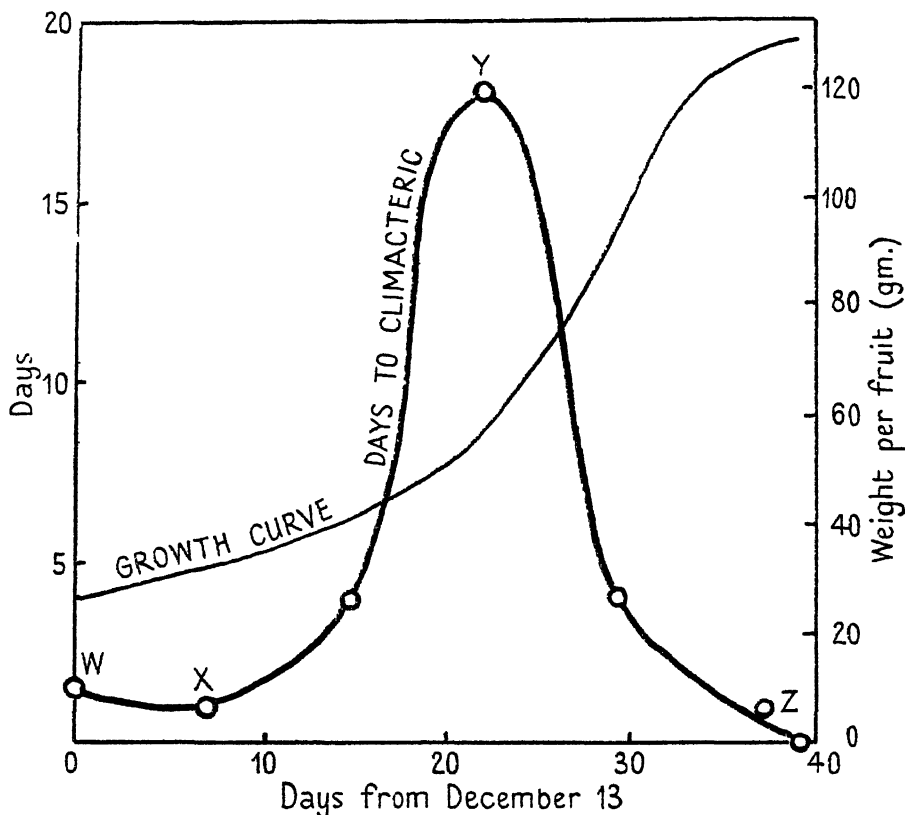


FIG. 5. The growth curve and the relation between 'maturity' and chronological age of peaches (Peregrine).

than either young or old fruits and exhibit maximum length of storage life. This stage of maximum longevity coincides roughly with the beginning of the period of maximum rate of increase in size of the fruit on the tree (see growth curves in Figs. 5 and 6).

Apart from the two phases comprising the major rise and fall ( $x-y-z$ ), some interest attaches to the early period when, both in peach and plum, there appears to be a shortening of the pre-climacteric interval ( $w-x$ ). The evidence is based only on the behaviour of the second sample in each case. The phenomenon may possibly be connected with the presence of the large stone characteristic of drupaceous fruits. In the case of the plum, Donen (1936) has shown

that the stone (endocarp plus embryo) attains its full size in mid-December, when it forms 8 per cent. of the total weight of the fruit. This figure falls to just over 1 per cent. in the fully grown plum.

The stone is larger in the peach, forming as much as 19 per cent. at the time of its maximum growth; and even in fully grown peaches it may still

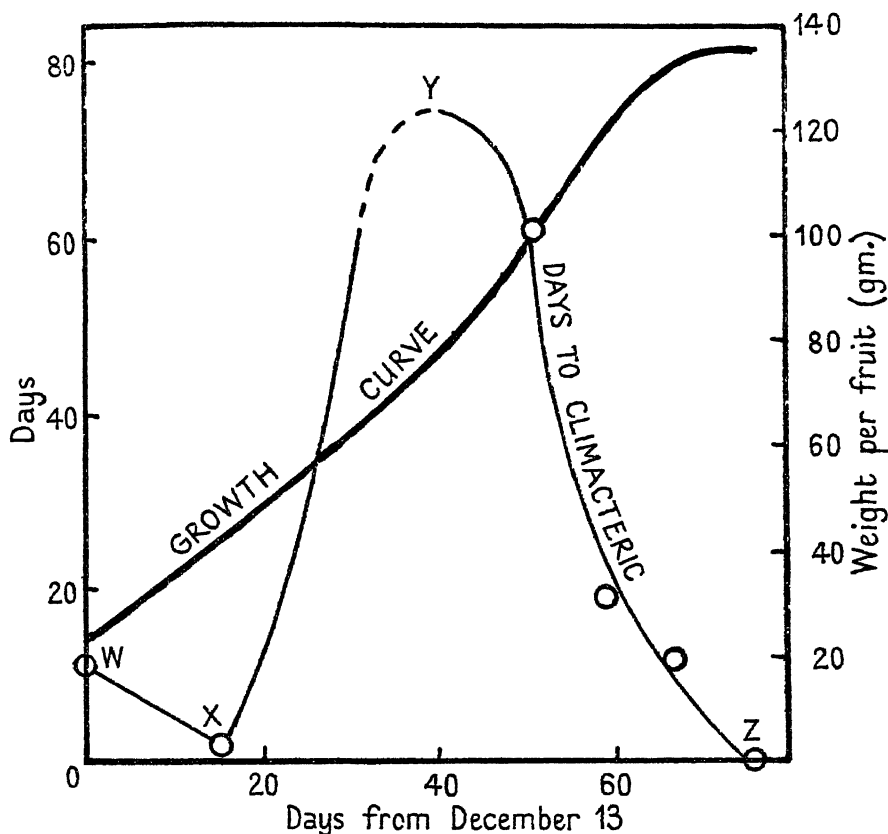


FIG. 6. The growth curve and the relation between 'maturity' and chronological age of plums (Kelsey).

represent over 6 per cent. of the total weight. It is only after the stone has attained its full size that there is a rapid increase in the rate of growth of the flesh, the period of cell enlargement. Previous to this, nutrients entering the fruit are nearly all absorbed by the growing stone. The 'predominance' of the stone ('seed') in the early stages of the growth of the peach has been emphasized by Lott (1932).

It is suggested that changes of a senescent character may make their appearance in the flesh at this period. But, with the diversion of nutrients from the stone and the rapid enlargement of the cells of the flesh, these senescent tendencies disappear and there is a drift away from metabolic 'maturity'.

The nature of the factors which initiate ripening and the climacteric rise in the respiration of plant tissues is very imperfectly understood. Earlier views which emphasized the starvation factor have largely been abandoned—cf. Kidd (1935), Wardlaw and Leonard (1936), Singh and Mathur (1936)—since it has been shown that fruits in the last stages of senescence very often still contain large quantities of apparently easily available sugars. In the case of young peaches and plums it might be possible to make out a plausible argument for starvation as the basal factor initiating the onset of the climacteric; for the cells are relatively rich in cytoplasm and poor in carbohydrate reserves. But the theory of starvation cannot account for the climacteric in fully grown peaches and plums, and it seems unlikely that the process of ripening is fundamentally different in fruits picked at different stages during growth, or is different in fruits as compared with other plant tissues such as leaves.

Kidd states that in apples gathered 'at the beginning of the second period of growth', i.e. the period of cell enlargement, 'the cytoplasm and the cane sugar *per cell* are at their minimum, and so therefore is respiratory activity. Fruit gathered at this stage will thus have a longer life during storage than fruit gathered during any subsequent stage. . . . Fruits of a short life-cycle, such as the strawberry, exhibit a higher respiratory activity than fruits of a longer life-cycle, such as the apple.' Similarly for the peach and the plum an inverse relationship may be demonstrated between length of life (measured by the length of the pre-climacteric interval) and the rate of respiration. This is seen not only in the lower rate of respiration of peaches and plums of intermediate age compared with fruits picked at an earlier or later stage; it is also demonstrated by the much longer life of the plum in store and its much lower rate of respiration as compared with the peach.

From this evidence it is clear that fruits which respire more rapidly die sooner; and, since respiration is known to involve the destruction of carbohydrate, it is natural to suppose that death ultimately results from the depletion of the supplies of respirable materials. As this conflicts with the fact that only a fraction of the carbohydrate reserves is actually consumed, some other hypothesis must be sought.

It is suggested that fruit when picked contains a limited amount of some substance which retards the onset of ripening and which is used up in some way during respiration and at a rate more or less proportional to the rate of respiration. Copisarow (1936) has put forward evidence to show that maleic acid acts as an inhibitor of ripening in fruits and vegetables and that it may be converted into ethylene which acts as an accelerator. An explanation of ripening in terms of inhibitors and accelerators seems more acceptable than any other hypothesis in the light of what is known at present.

#### SUMMARY

1. The  $\text{CO}_2$  respiration at  $25^\circ \text{C}$ . of peaches and plums picked at intervals during growth was determined.

2. It is shown that in both cases the very young fruits have an early and pronounced climacteric. Fruits of intermediate age have a very much delayed climacteric and exhibit the maximum longevity in store.
3. The stage of maximum length of storage life coincides with the beginning of the period of most rapid increase in size of the fruit on the tree.
4. There is an inverse relation between longevity and the rate of respiration.
5. The nature of the respiratory climacteric is discussed.

The writer wishes to thank the Senate of the University of Capetown for the grant of the Smartt Memorial Scholarship, which enabled this work to be carried out.

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# The Salt Relations of Plant Tissues

## I. The Absorption of Potassium Salts by Storage Tissue

BY  
WALTER STILES  
AND  
A. D. SKELDING

With eleven Figures in the Text

### INTRODUCTION

THE investigations to be described in this series of papers are a development of work on the permeability of plant cells carried out by one of us in conjunction with I. Jørgensen some twenty-five years ago. The object of such investigations on the permeability of plant cells, using this term in a wide sense, is to determine the laws governing the passage of materials into and out of cells and, as far as possible, to explain these laws in physico-chemical terms. It is clear that these problems lend themselves to diverse methods of attack and that information of value for their solution may be obtained by an examination of a variety of different processes. Thus the diffusion of solutes in various colloidal systems, the diffusion of water and dissolved substances through membranes, and the effect of various substances on protoplasm are all phenomena which may have a bearing on this subject. But most necessary and important of all is the obtaining of actual data of the absorption of substances by living cells. A mass of qualitative information regarding the entrance into plant cells of acids, alkalis, inorganic salts, and a variety of organic compounds has been available for many years. Quantitative data, on the other hand, are scanty, yet without such data it is impossible to obtain any complete understanding of the mechanism of the permeability of cells.

This series of papers is concerned with observations on the absorption of inorganic salts by plant tissue. From a physiological point of view such salts, or rather the ions composing them, fall roughly into three groups. The first comprise nutrient ions, those which enter into the composition of the plant body or which comprise or contain essential elements for plant growth and which include the kations K,  $\text{NH}_4$  (for nitrogen), Ca, Mg, Fe (both ferrous and ferric), and the anions  $\text{SO}_4$  (for sulphur),  $\text{NO}_3$  (for nitrogen), and  $\text{H}_2\text{PO}_4$ ,  $\text{HPO}_4$ , and  $\text{PO}_4$  (for phosphorus). To these must be added, for some plants

[Annals of Botany, N.S. Vol. IV, No. 14, April 1940.]

at any rate, Mn and  $\text{BO}_3$  and other anions containing boron and manganese. A second group contains ions which, while not generally regarded as essential for plant nutrition, exert no conspicuous toxic action in low concentrations; among such ions are Na, Al, Cl, and  $\text{SiO}_3$ . The third group comprises definitely poisonous ions such as the kations Ag, Cu, Zn, Hg, and the anions CN, CNS. The distinction between these three groups is not sharp. Thus even nutrients may have a toxic action if presented to plant tissue in too high a concentration. Again, it would appear that for some plants sodium and chloride are essential for adequate growth, while there is evidence that in some cases zinc and copper, in very small amount, may be essential elements. Indeed, it is not by any means certain how far a number of elements not usually included among plant nutrients are actually necessary in small quantity for plant growth, and the number of so-called micro-nutrients or trace elements may be greater than has generally been supposed.

It is our intention to obtain quantitative data of absorption by various kinds of plant cells of salts of these different groups. Only by so doing can we hope to obtain the information which will enable us to formulate the laws governing the passage of inorganic substances into and out of plant cells. In the first instance we propose to deal with storage tissues.

#### THE PRESENT STATE OF KNOWLEDGE

Although there are a number of rather isolated observations on the absorption of the ions of one salt or another by such tissues, and although in recent years much has been written on this question, any general quantitative information regarding this problem is largely wanting. Indeed, as far as we are aware, the only attempt that has been made to obtain quantitative and comparative information on the absorption of a variety of salts by storage tissue was that made by Stiles and Kidd (1919 *a, b*) some twenty years ago in which the absorption of a number of inorganic salts was followed indirectly by observing the fall in electrical conductivity of the external liquid. This work was in the nature of a pioneer research, and, as these authors fully realized, the method used could only yield approximate data. Nevertheless, it established the general relationship between absorption and concentration of salt, namely, that absorption from weaker solutions is greater, relative to the concentration, than from stronger solutions of the same salt. Indications were also obtained of the relative rates and total quantities absorbed of different ions by the same tissue. For reasons stated at the time it is clear that changes in electrical conductivity cannot yield precise information of the amount of any particular ion absorbed from a solution, but although occasional attempts have been made subsequently to follow the course of absorption of ions by other methods, exact knowledge of the quantitative relations of inorganic salts and their ions remains scanty. One of the greatest obstacles in the way of obtaining this desired information has been the difficulty of measuring, even approximately, small quantities of the ions concerned. Chemical analytical

methods have subsequently been employed for this purpose, but the degree of accuracy when dealing with even moderately dilute solutions, such as those of 0.01 M concentration, is often not high, while with more dilute solutions such methods are generally inapplicable. Attempts have also been made to use the rate of deplasmolysis as a measure of entry of salt into the cell, but recorded results are few, while the degree of accuracy is not high, even if the results observed by this method are rightly interpreted.

Apart from the early investigations to which reference has already been made, as far as we are aware the only recorded observations in which the course of absorption of the actual ions of salts by storage tissues has been followed are those of Stiles (1924), Asprey (1933, 1937), and Steward (1932, 1939).

Stiles followed the course of absorption of both ions from 0.02 N solution of ammonium phosphate by carrot, parsnip, and beetroot and from 0.1 N solution of sodium chloride by artichoke tuber and carrot root, by direct chemical analysis of the external solution. In each case there was inequality in absorption of the two ions of the salt, the kation being absorbed more rapidly, and to a greater extent when the experiment was stopped, than the anion, while the same general relation between concentration and absorption was observed in each case. Evidence was obtained that the excess absorption of kation over anion was compensated by the exosmosis of other ions from the tissue.

Asprey (1933 *a*) examined the absorption by potato tissue of calcium and ammonium from 0.02 N solutions of their chlorides over comparatively short periods, about 10 hours in the case of calcium chloride and 24 hours in the case of ammonium chloride. In a third experiment the absorption by potato of both ions of ammonium chloride was followed over a period of 9 hours. The more rapid absorption of the kation was also observed here. In a later paper Asprey (1937) examined the course of absorption of the ions of ammonium chloride by potato tissue after the latter had been washed in water for different lengths of time, namely, 24, 72, and 120 hours. In each case the excess absorption of kation over anion was again observed, but it was also found that the absorption of both ions was increased by extending the washing period.

The published contributions to our knowledge of the course of salt absorption by storage tissue made by Steward and his collaborators consist of two or three experiments in which the absorption by potato tuber of bromide from potassium bromide was followed, and in one of which the concentration of potassium in the sap was also measured on four successive days, and another in which the absorption of both ions of rubidium bromide from a 0.002 N solution was examined. In both cases inequality of absorption of the two ions of the salt was evident. Steward and Harrison claim that in the case of rubidium bromide only the rubidium ion is absorbed in appreciable amount for two days, after which both ions are absorbed at the same rate, and that,



moreover, this rate remains constant for at least three days. According to Steward and Berry the bromide ion is absorbed at a constant rate from potassium bromide also.

The observations so far made on the course of absorption of definite ions by storage tissue are summed up in the following table.

TABLE I  
*Previous Observations on the Course of Absorption of Ions  
by Storage Tissue*

Salt.	Initial concentra- tion.	Tissue.	Length of experiment in hours.	Observer.
Ammonium phosphate	0.02 N	Carrot root	49	Stiles, 1924
"	"	Parsnip root	47	"
"	"	Beetroot	48	"
Sodium chloride	0.1 N	Artichoke tuber	47	"
"	"	Carrot root	126	"
Calcium chloride*	0.02 N	Potato tuber	24	Asprey, 1933
Ammonium chloride	"	"	24	"
"	"	"	25	Asprey, 1937
Potassium bromide	0.000539 N	"	99.5	Steward, 1932
" †	0.00075 N ‡	Artichoke tuber	138	Steward and Berry, 1933
Rubidium bromide	0.002 N	Potato tuber	95.5	Steward and Harrison, 1939

\* Kation absorption only examined. † Anion absorption only examined.  
‡ Solution renewed daily.

In addition there are a number of observations by Stiles, Asprey, and Steward and his collaborators on the amount of one ion or both ions of a salt absorbed after a certain time which serve to indicate certain points regarding salt absorption, but as far as we are aware all the data published so far on the course of absorption of individual ions by storage tissue are included in the table. They indicate clearly the fragmentary state of our knowledge of the subject. The absorption of six salts has been examined and only one concentration of each salt has been employed, while the concentration has varied from 0.1 N in one case to 0.000539 N in another. Different tissues have been used in different experiments, while the length of time over which absorption has been measured varies from 24 to 140 hours. On account of these differences the collation of the results of different workers requires a certain amount of care, and failure to take account of these differences has, in our opinion, led Steward to certain unjustified criticism of the work of others to which reference will be made later in this paper.

In view of the present very fragmentary state of knowledge, we consider that the first necessity towards obtaining an insight into the salt relations of storage tissues is a general survey of the course of absorption of a wide range of salts presented to tissues of various kinds in different concentrations. The

present paper constitutes the first instalment of this survey and gives the results of investigations on the absorption by carrot-root tissue of a number of potassium salts used in a range of concentrations.

#### FACTORS AFFECTING SALT ABSORPTION

Although our knowledge of the quantitative aspects of salt absorption by storage tissues is so fragmentary, it is well established that this absorption is influenced by a number of factors. These are as follows.

1. *Nature of the tissue.* It is to be expected that tissues of different species or varieties will exhibit differences in their absorption of salts. Examples of this are given by Berry and Steward (1934), who conclude that one reason for such differences is the presence or not in the tissue of many cells capable of renewed growth.

2. *Space relations of the tissue.* The rate of absorption per unit volume is very definitely affected by the thickness of the tissue, or, put in more general terms, by the relation of surface to volume of the tissue. Some early experiments of Ruhland (1909) showed this, and it has been confirmed more recently by Steward, Wright, and Berry (1932).

3. *History of the tissue in the plant.* It is conceivable that the conditions under which the plant yielding the tissue has grown may affect its capacity for the absorption of any particular salt. If, for example, a plant is grown on a medium rich in any particular mineral constituent it is possible that the tissues of such a plant may contain more, and, in consequence, absorb less of that constituent, than the tissues of a plant of the same species or variety grown on a soil poor in that salt.

4. *History of the tissue after separation from the plant.* The treatment of the tissue after its removal from the plant and before the actual measurement of its absorption of salt may considerably influence this absorption. Thus it is usual in experiments of this kind to wash the tissue in water for a certain time after its separation from the storage organ containing it. Now Asprey (1937) has shown that the absorption of the ions of ammonium chloride by potato tuber tissue is definitely increased, both in regard to its initial rate and the amount absorbed after 24 hours, with increase in this preliminary washing period, although no such effect could be detected with artichoke tuber. Asprey (1933 *b*) has also shown that pre-treatment of potato tuber tissue with solutions of alkali chlorides increases subsequent absorption of ammonium from ammonium chloride, while pre-treatment with calcium, magnesium, and aluminium chlorides brings about a decrease in the absorption of the ammonium ion as compared with that which occurs after treatment with distilled water.

5. *Nature of the salt.* The same tissue absorbs different salts or their constituent ions at different rates and so to different extents in the same time. This was clearly shown in the pioneer experiments of Stiles and Kidd (1919 *b*).

6. *Concentration of the salt.* The great effect of concentration of the salt on

its intake was shown by Stiles and Kidd in 1919. Although the actual rate of intake of the salt or its ions is greater the higher the concentration of the salt, actually the rate of absorption relative to the concentration decreases with increase in concentration.

7. *Presence of other substances in the medium surrounding the tissue.* There exists a considerable mass of data concerning the effect of the presence of one substance on the absorption of another. In most cases the presence of a second substance in solution reduces the rate of entry of another, a phenomenon to which the term antagonism is applied. In the case of storage tissues such an effect has been observed by Asprey with potato tuber, the absorption by which of the kations ammonium and calcium from their chlorides is generally reduced by the presence of the chloride of another metal in the solution.

8. *Supply of oxygen.* The importance of a supply of oxygen to maintain the vitality of tissue kept in an aqueous medium was shown by one of us in 1927. It was then shown that storage tissue kept immersed in still and unaerated water at 20° C. dies in two or three days, whereas if the tissue is kept shaken in water and if the oxygen supply is renewed at intervals, the tissue will retain its vitality for three or four weeks. Since then Steward and his collaborators have shown that the amount of the ions of potassium bromide absorbed by storage tissues after periods of from one to three days is lowered if the supply of oxygen to the tissues is reduced below a certain amount.

9. *Temperature.* It is to be expected from what is known of the effects of temperature on other plant processes that, within limits, increase in temperature will bring about an increase in the rate of salt absorption. Both Petrie (1927) and Steward (1932) have published data of the amount of absorption of ions at different temperatures after a single interval of time, and these data indicate that between 3° and 23° the rate of absorption is definitely increased by rise in temperature. In referring to the temperature coefficient ( $Q_{10}$ ) of 2.2 obtained by Stiles and Jørgensen for the absorption of hydrogen ions by potato tuber tissue, Steward, from a single determination of bromide absorption at each of four different temperatures, indeed remarks that it is 'only between 14° C. and 20° C. that a temperature coefficient of this order is obtained'. But Steward is in error in supposing that it is possible to calculate temperature coefficients from single determinations in this way. To express the effect of temperature on salt absorption as a temperature coefficient it is necessary to determine the course of absorption at each temperature and compare the rates of intake at the same stage of intake at the different temperatures.

From this brief survey of the factors affecting salt absorption by storage tissue it is clear that for the present investigation, in which the absorption of a number of salts in different concentrations is the subject of examination, care must be taken to standardize as far as possible the other conditions of the experiment which may affect the course of ionic intake. These are the degree of uniformity of the tissue (cf. factors 1 and 3 above), uniformity of

treatment in the preparation of the tissue (factors 2 and 4), and control of oxygen supply and temperature. The means of dealing with these questions is dealt with in the next section of this paper.

#### MATERIAL AND METHODS

The tissue used in these experiments consisted of carrot root cut in discs 2.0 cm. in diameter and 0.1 cm. thick, prepared as described in earlier papers (cf. e.g. Stiles and Stirk, 1931). After cutting, the discs were washed in running tap-water for 16 hours and then in distilled water for 2 hours. They were well mixed and then transferred in batches of 60 to stoppered bottles of 420 ml. capacity each containing 200 ml. of solution. Each experiment was conducted in triplicate. By this procedure variation in the different samples of tissue was minimized as was also variation in the rate of absorption related to different periods of preliminary washing.

Uniform treatment of the tissue with regard to the supply of oxygen was achieved by the very simple device of perforating the stopper of each bottle containing tissue with a hole 1 cm. in diameter. The atmosphere in the bottles was thus kept in connexion with the outside air, while diffusion shells inside the bottles were broken down by keeping the bottles continuously shaken by a mechanical shaker of the trolley pattern.

It has been repeatedly suggested by Steward that the technique employed by other workers in which the tissue was contained in stoppered bottles the atmosphere in which was only brought into contact with the outer air at intervals involves the production of anaerobic conditions, or at least oxygen shortage, in the tissues, and that absorption of salt is restricted or even suppressed altogether on this account. While the importance of oxygen supply in the salt relations of plant tissues is beyond question, there can be no doubt that in many of the experiments to which Steward has raised this objection there was not, in actual fact, such a shortage of oxygen as Steward suggests. This has, indeed, already been rightly pointed out by Asprey (1937), and as long ago as 1927 one of us had shown that tissue kept in the conditions to which Steward objects will maintain its vitality for three or four weeks, whereas under really anaerobic conditions the tissue is dead within a day or two. In order to maintain an adequate oxygen supply Steward used bottles of large capacity (2 litres) filled with solution and containing a comparatively small amount of tissue (60 discs 3.4 cm. in diameter and 0.75 mm. thick) and passed a continuous current of air or other gas mixture containing oxygen through the liquid. It is open to question whether the oxygen concentration at the surface of the discs in such a system is higher, or even as high, as in a system where there is in the bottle a reserve of air which is brought into contact at intervals with the outside air, at any rate where the experiment is of comparatively short duration, as in the case of Asprey's, and where the connexion with the outside air is made at fairly frequent intervals and where the system is kept continually shaken.

In order to determine whether our technique did indeed lead to a depression in the rate of salt absorption as compared with that devised by Steward, we repeated in part a recent experiment by Steward and Harrison in which the absorption of rubidium bromide by potato tissue was followed. We used the same concentration of rubidium bromide, namely 0.002 N, as these workers, and determined the fall in halide concentration of the external solution by the method mentioned later. The temperature of our experiments was, however, a little lower than that used by Steward and Harrison, namely, 21° instead of 23° C. The results are summarised in Table II.

TABLE II  
*Absorption of Bromide from 0.002 N Rubidium Bromide  
by Potato Tuber Tissue*

Time (hrs.).	Concentration of halide in external solution (initial concentration taken as unity)		
	Expt. 1.	Expt. 2.	Expt. 3.
4.2	1.052	1.058	1.039
8.3	0.988	0.962	1.060
26.0	0.986	0.986	1.073
50.5	0.924	0.793	0.927
71.0	0.725	0.655	0.793
93.7	0.562	0.493	0.627

These data indicate the change in halide concentration of the solution external to the tissue. If there is any exosmosis of chloride from the tissue the actual absorption of bromide would be greater than the values indicated in the table by the amount of this exosmosis.

A determination of the water content of fully imbibed potato tissue showed that this amounted to 84.75 per cent. of the fresh weight. Since the volumes of solution and tissue in each experiment were approximately 200 and 18.7 c.c., it follows that the concentrations of bromide in the tissues after absorption had proceeded for 93.7 hours, expressed as mg. equivalents per 1,000 gm. water in the tissue, are not less than 11.05, 12.8, and 9.4. The absorption in Steward and Harrison's experiment after this time, as given by their time absorption curve, is 14.8.

When we consider that (a) our values have to be raised by the amount of any exosmosis of chloride, (b) our experiments were conducted at a temperature 2° C. lower than that of Steward and Harrison, and (c) that owing to the greater volume of solution relative to that of tissue in the experiments of Steward and Harrison as compared with ours the tissue in the former experiment was for the greater part of the time absorbing from a stronger solution than in our experiments, we are justified in concluding that there is no depression of absorption resulting from our experimental arrangement as compared with that elaborated by Steward. Moreover, it must be pointed out that even if conditions for absorption are not the optimum it does not follow that completely valid and important conclusions cannot be drawn from the experimental

results obtained. In any case the conditions in such experiments are artificial and not natural ones and the important point is the definition of the conditions. It is only with the advance of knowledge that realization comes of what are, and what are not, the essential factors in any particular experiment.

The remaining factor which is known to influence salt absorption is temperature. In our experiments this was maintained at  $21^{\circ}\text{C}$ . by an electrically operated thermostatic arrangement employing the bi-metal type of regulator supplied by Messrs. Sun-Vic Controls Ltd.

The potassium salts used in the present investigation were the chloride, bromide, nitrate, dihydrogen phosphate, and sulphate. In all cases the salts were of the Analar grade supplied by British Drug Houses. Absorption from these salts in a range of concentrations was examined, the usual range of initial concentrations being 0.02 M, 0.005 M, and 0.001 M. The intake of both ions of each salt was determined by the fall in concentration of the ion in the external medium, small samples of the solution being removed for analysis at intervals, a quantity of tissue being removed at the same time in order to keep the relative quantities of solution and tissue constant. This method of following ion intake is, in our opinion, superior to the alternative method of analysing the expressed sap for three reasons, namely, (1) the tissue used throughout one experiment remains practically constant so that differences due to variations in different batches of tissue do not confuse the results; (2) considerably less tissue and solution are necessary for one experiment, thus materially reducing the bulkiness of apparatus; (3) the solutions to be analysed contain so little impurity that with the analytical methods employed the ions can usually be determined directly, whereas the extraction and purification of sap for analysis is a lengthy process. The difficulty of obtaining really reliable samples of expressed sap is well known and has often been noted in the literature of the subject. We are aware that Steward has alleged that the method of analysing expressed sap is superior to that of analysing the external solution, but he gives no reason for his assertion and we cannot agree with him if his statement is to be regarded as of general application. It may be claimed that an advantage of analysing sap instead of solution is that higher concentrations of the ions to be determined are usually to be found in the sap than in the external solution. But even this is not necessarily always the case, while modern methods of analysis are now available which allow the determination with a reasonable degree of accuracy of very low concentrations. We have no doubt that the accuracy of such determinations of ions in the external solution is certainly not less than that of determinations of ions in the expressed sap. But where, as in Steward's system, the rate of fall of concentration of the external solution may be slow, analysis of sap may give more satisfactory results. This would definitely not be the case with our experimental arrangements.

Indeed, the chief reason for the scantiness of data on salt absorption is to be found in the difficulty of estimating the small quantities of material usually

available for analysis in work of this kind. Of recent years, however, the estimation of very small quantities of most inorganic ions has been made possible by the development of new methods. Five methods in particular are proving of great value in this connexion: these involve the use of (1) adsorption indicators, (2) the colorimeter and its variant, the absorptiometer, (3) the polarograph, (4) the spectrograph, and (5) radioactive isotopes. By these methods it is possible in some cases to measure quantities as small as a  $\gamma$  or even less, although in other cases such a sensitivity can scarcely be reached. In the present investigation we have used the first three of the methods as indicated below.

Potassium has been determined throughout by the polarograph. Although this instrument has been used very little in this country, so many accounts of it have now been published that a further description is unnecessary. We would here simply refer to the descriptions of Heyrovský (1936), Prát (1928), and Hohn (1937), and point out that the measurement of the strength of a particular kation depends on the fact that when a gradually increasing potential difference is applied to a solution of a salt containing the kation in presence of another more electronegative kation in considerable excess, a current flows when a definite potential difference is reached depending on the nature of the kation, while the strength of the current depends on the concentration of the kation. The proper conditions for the development of this current were worked out by Heyrovský and his collaborators. One of the most important is that the kathode should be in the form of falling drops of pure mercury, while the anode consists of a layer of mercury with a relatively large surface. The determination of potassium can be accomplished by this means if the solution contains an excess of a salt of tetramethyl ammonium or tetraethyl ammonium. We used a 0.1 M or 0.01 M solution of tetraethyl ammonium chloride for this purpose, the weaker strength being used to determine concentration of potassium of 0.001 M or less. For the determination of potassium special electrode vessels were used through which a current of hydrogen could be passed, and in all potassium determinations air was driven out of the electrode vessel by a stream of hydrogen. The values of potassium obtained in this way will include any sodium or ammonium ions which have diffused from the tissue into the external solution. We were, however, unable to detect any of these ions in the external solution in any of our experiments and we regard the values obtained as giving reliable data of the potassium ion concentration of the solutions examined. With careful pipetting of the solutions and the use of purified and re-distilled mercury the potassium determinations are probably correct to 1 or 2 per cent.

Chloride and bromide in the solutions of an initial concentration of 0.02 M were determined by means of adsorption indicators. A 5 ml. sample of the solution was titrated with standard silver nitrate for both chloride and bromide. In the case of chloride the indicator used was a 0.1 per cent. solution of dichlorofluorescein in 60 per cent. alcohol, a few drops of which were added

to the sample just before the end-point was reached. The colour change at the end-point was from a yellow-green to red. For bromide the indicator is a 1 per cent. solution of eosin in water and the end-point is indicated by a change from crimson to a rose-red colour. These methods may be used, with such small samples, down to a concentration of 0.005 M only, and so were employed for the 0.02 M samples only.

Since with lower concentrations of halide the use of adsorption indicators was not suitable, the determination of chloride and bromide in solutions of initial concentration of 0.005 M or less were made by the polarographic method as described by Revenda (1934).

For this purpose a dropping mercury *anode* is used and a large mercury surface as *kathode*, while a solution of potassium nitrate was used as a ground liquid. For chloride the method is quite satisfactory, but with bromide there is the disadvantage that the depolarizing potentials of chloride and bromide are so close together that any chloride which diffuses out of the tissue raises the apparent concentration of bromide in the external solution so that the absorption of bromide is actually greater than its polarographic determination would suggest. There is evidence of such an exosmosis of chloride from the tissue, although this chloride is subsequently reabsorbed, so that the curves for bromide absorption may be somewhat distorted, especially over the first part of their course.

Nitrate was also determined by the polarograph as described by Tokuoka and Růžička (1934). Here a dropping mercury *kathode* has to be used and the nitrate must be in the presence of a considerable excess of a lanthanum salt and is best determined in an atmosphere of hydrogen. We are indebted to British Drug Houses for specially preparing for us lanthanum chloride free from nitrate.

The determination of phosphate was carried out by means of a Spekker absorptiometer in the manner described by Berenblum and Chain (1938). This method is particularly sensitive and quantities of a  $\gamma$  were determined with ease.

Sulphate was determined by titration with a standard solution of barium chloride using tetrahydroxyquinone as an adsorption indicator as described by Schroeder (1933). The samples used were of 15 or 20 ml. volume and were first rendered just acid to phenolphthalein by the addition of 0.02 N hydrochloric acid or caustic soda as required. 25 ml. of isopropyl alcohol were then added and a quantity of solid tetrahydroxyquinone. 80 mg. of the indicator were used for samples containing less than 100 p.p.m. of sulphate and 150 mg. for those containing between 100 and 2,000 p.p.m. After mixing to dissolve the indicator the sample was titrated until the colour changed from yellow to rose red. The end-point was made sharper by adding about 1 ml. of 0.1 N silver nitrate solution towards the end of the titration when an excess of chloride was present in the sample. After some experience of the colour change this method may be used to estimate the sulphate in solutions ranging



in concentration from 40 to 2,000 mg. per litre. Stronger solutions were diluted to concentrations within this range.

### EXPERIMENTAL RESULTS

#### 1. *Potassium chloride.*

Three series of experiments were made with potassium chloride with different methods of air-supply. In one the usual routine already described was used, that is, the tissue was contained in standard bottles with a hole in the cover and was continuously shaken; in the second the arrangement was similar except that the stoppers of the bottles were without the hole, so that contact was made with the outer air only when samples of liquid were removed for analysis, while in the third the bottles were kept still but the liquid was aerated by a current of air for a period of 10 minutes every hour by means of a pump which was brought into action at regular intervals by means of a time switch.

In the first series of experiments three concentrations of potassium chloride were used, namely, 0.02 M, 0.005 M, and 0.001 M. The course of absorption of potassium and chloride as calculated from the fall in concentration of the external solutions is shown in Table III. In all cases the values are given as proportions of the original amount in the external solution so that an absorption of 0.5 indicates that half of the ion originally present in the external solution has been absorbed by the tissue.

TABLE III

#### *Course of Absorption of Potassium Chloride by Carrot Root Tissue*

Initial concentration.	Time (hrs.).	Absorption	
		K.	Cl.
0.02 M	18.9	0.13	0.11
	45.5	0.26	0.19
	91.1	0.37	0.36
	114.8	0.45	0.45
0.005 M	0.67	0.09	0.07
	18.67	0.40	0.40
	45.75	0.62	0.745
	90.8	0.805	0.85
	114.4	0.91	0.93
0.001 M	0.5	—0.05	0.06
	18.5	0.35	0.60
	45.1	0.56	0.80
	90.7	0.70	>0.98
	114.3	0.88	1.0 approx.

Various points regarding the absorption may be noted. In the first place both ions are continuously absorbed throughout the whole period of the experiment, with the exception that in the lowest concentration an exosmosis

of potassium takes place from the tissue at the beginning of the experiment, although within the next 18 hours the movement of potassium is reversed and absorption takes place. Secondly, the absorption is considerable, the chloride being practically completely removed from the 0.001 M solution. Thirdly, as previously observed, the absorption relative to the concentration increases

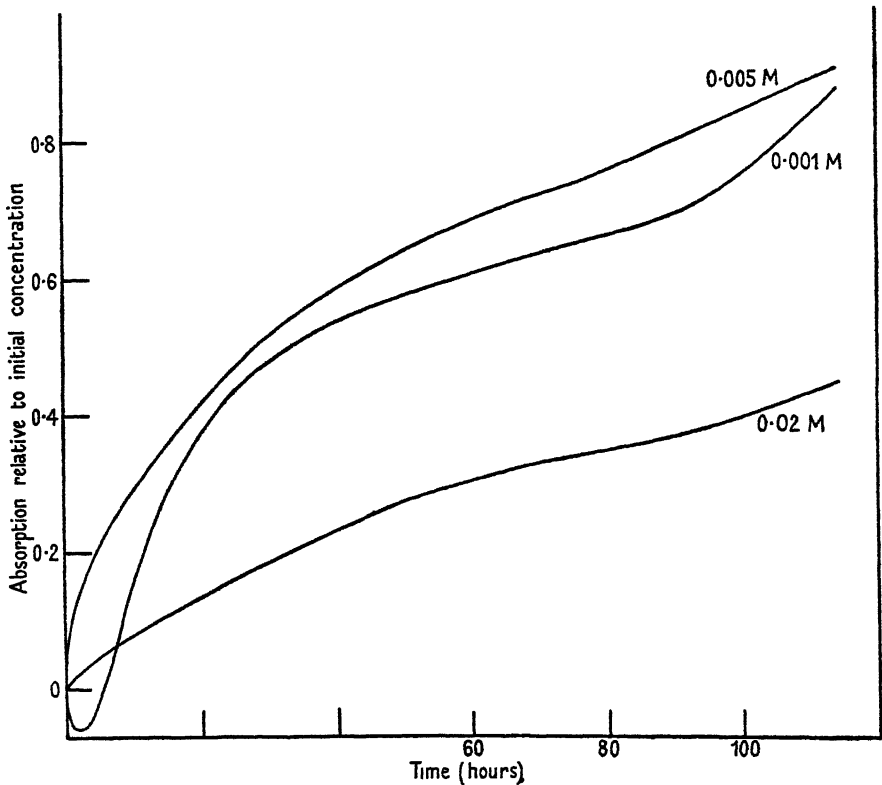


FIG. 1. Curves showing the course of absorption of potassium by carrot tissue from solutions of potassium chloride having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

with dilution, apart from the complication resulting from exosmosis of potassium in the weakest solution. It will be noted that when storage tissue is placed in distilled water there is an exosmosis of electrolytes (Stiles, 1927) which we have found by polarographic analysis includes potassium ions. It would appear that there is still this tendency for exosmosis when the liquid surrounding the tissue is a potassium salt. It may be noted also that if this exosmosis is independent of the concentration of the external solution, in a higher concentration of potassium this tendency will reduce the absorption relative to the concentration less than in a weaker concentration. This

probably accounts for the relative absorption of potassium being somewhat higher from a 0.005 M solution than from a 0.001 M solution.

In previous work considerable attention has been directed to the unequal absorption of the two ions of a salt. It will be observed that in the experiments described here the difference is not great, indeed, sometimes non-existent, particularly in later stages of absorption. The difference, indeed, appears to

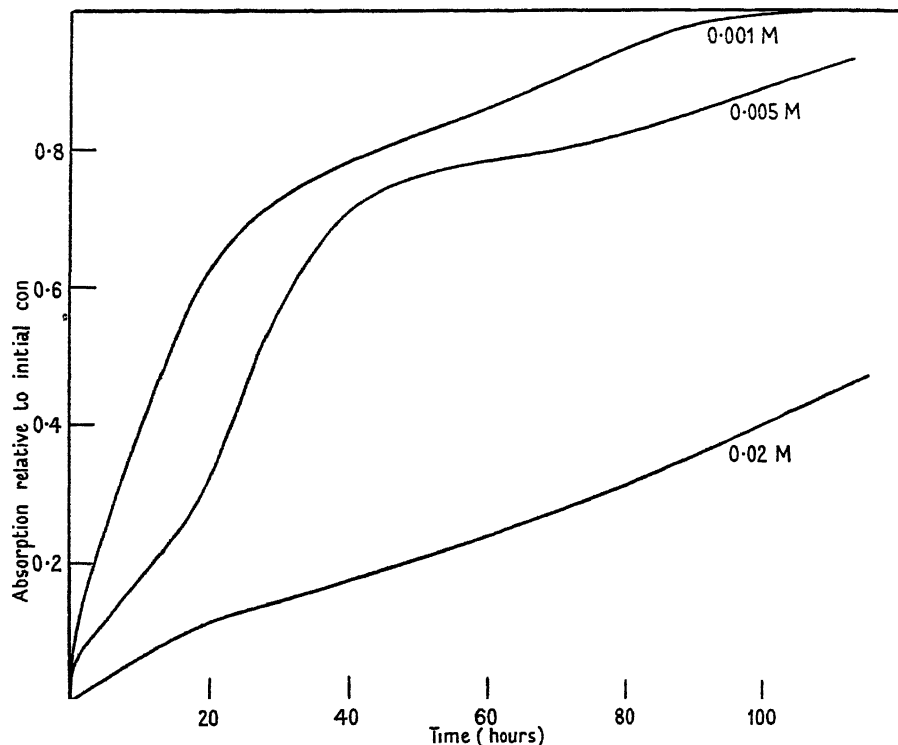


FIG. 2. Curves showing the course of absorption of chloride by carrot tissue from solutions of potassium chloride having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

be largely related to the exosmosis of potassium in the early stages of the experiment.

The results of these experiments are shown graphically in Figs. 1 and 2. It is at once clear that the course of absorption is not a simple one. In the case of the kation no less than three phases may be observed: (1) an initial phase of negative absorption (exosmosis) here present in only the lowest concentration, followed by (2) a period of rapid absorption falling off with time, which gives place after about three days to (3) an increase in the rate of absorption, and this in spite of the very much lowered concentration of the external solution. This rate must ultimately fall on account of the exhaustion of the salt

in the external solution. The absorption of the anion follows a similar course except that there is not observed in any case an initial exosmosis of chloride.

In the second series of experiments with potassium chloride in which the tissue was kept continuously shaken in stoppered bottles, four concentrations of the salt were used, namely, 0.02 M, 0.005 M, 0.001 M, and 0.0002 M. The results are summarized in Table IV.

TABLE IV

*Course of Absorption of Potassium Chloride by Carrot Root Tissue*

Initial concentration.	Time (hrs.).	Absorption	
		K.	Cl.
0.02 M	0.5	0.012	—
	2.33	0.015	—
	8.1	0.062	0.049
	25.5	0.089	0.135
	50.3	0.104	0.17
	74.25	0.25	0.24
	92.4	0.32	0.35
0.005 M	0.33	0.017	—
	2.1	0.044	0.0395
	7.8	0.04	0.105
	25.3	0.19	0.30
	50.1	0.37	0.56
	74.4	0.57	0.75
	92.5	0.64	0.85
0.001 M	0.5	—	0.059
	2.2	0.013	0.0665
	7.25	—0.110	0.104
	24.8	0.084	0.43
	49.6	0.30	>0.9
	74.1	0.37	1.0 approx.
	91.9	0.45	1.0 approx.
0.0002 M	0.5	—0.28	—0.15
	2.2	—0.355	—0.079
	7.2	—0.405	—0.18
	24.8	—0.98	0.215
	49.3	—1.08	>0.9
	74.1	—1.46	1.0 approx.
	92.0	—0.76	1.0 approx.

The results for the first three concentrations are very similar to those recorded for the previous series. In the weakest solution, which was not used in the first series of experiments, exosmosis of potassium was much more marked, and although by the end of the experiment, i.e. after 92 hours, absorption of potassium had begun, the concentration of this ion in the solution was still 75 per cent. higher than at the beginning. There was also exosmosis of an anion, probably chloride, from the tissue into this weakest solution, but this was followed by absorption which was considerable even

after 24 hours, while after two or three days practically the whole of the chloride had been absorbed. Results of this series of experiments are similar to those previously described, except that the initial phase of the absorption reached a lower value and reached it earlier.

The third series of experiments with potassium chloride was carried out at the same time and with discs from the same stock of carrot tissue as those used

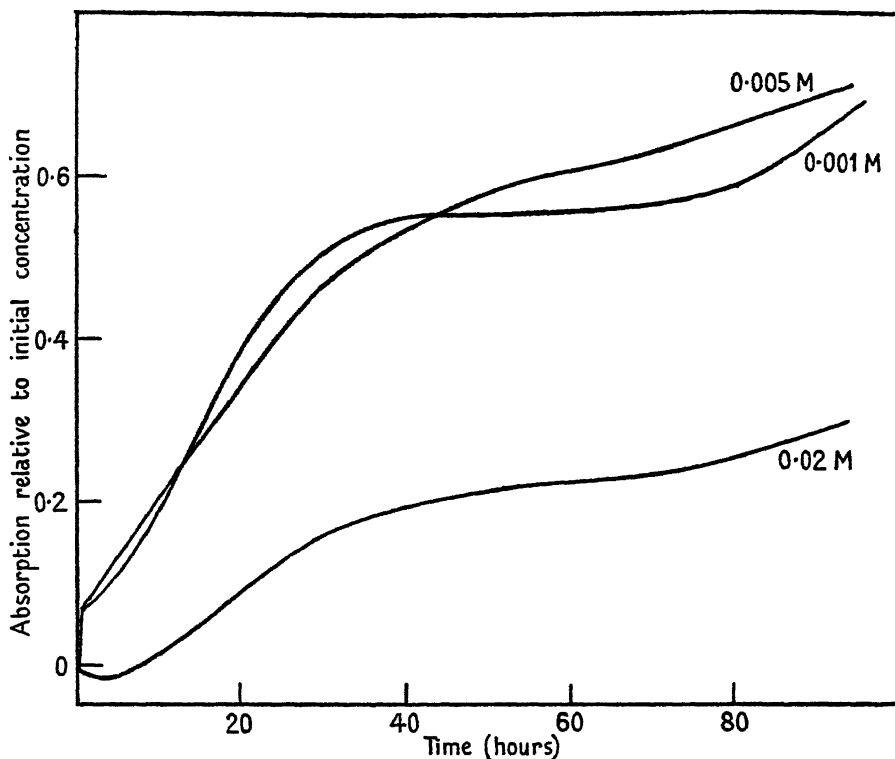


FIG. 3. Curves showing the course of absorption of potassium by carrot tissue from solutions of potassium bromide having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

for the second series, and the same four concentrations of salt were employed. The results are shown in Table V. It will be observed that the course of absorption was similar to that in the two other series of experiments with potassium chloride, but that the rate of absorption was somewhat less. Evidently intermittent aeration is less effective in speeding up absorption than continuous shaking.

At the same time it will be observed that although the rate of salt intake is less than in the two series of experiments previously described, just the same conclusions are to be drawn regarding the course of absorption of the two ions and the relationship of absorption to concentration.

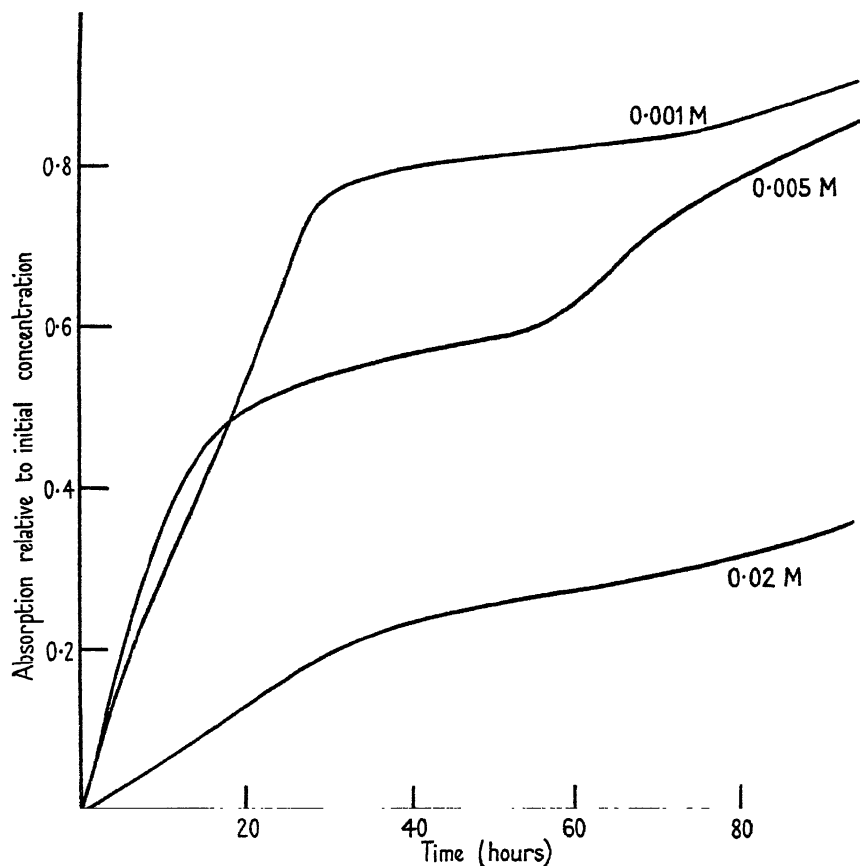


FIG. 4. Curves showing the course of absorption of bromide by carrot tissue from solutions of potassium bromide having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

TABLE V—*Absorption of Potassium Chloride by Carrot Tissue*  
(Solutions aerated intermittently)

Initial con- centration.	Time (hrs.).	Absorption		Initial con- centration.	Time (hrs.).	Absorption	
		K.	Cl.			K.	Cl.
0.02 M	0.5	0.012	—	0.001 M	0.5	—0.25	—0.037
	2.3	0.035	—		2.2	—	0.015
	8.1	0.044	0.0165		7.25	—	0.078
	25.5	0.042	0.074		24.8	—0.66	0.156
	50.3	—	0.15		49.6	0.011	>0.9
	74.25	0.16	0.19		74.1	0.27	1.0 appr.
	92.4	0.22	0.23		91.9	0.37	1.0 appr.
0.005 M	0.33	0.023	0.015	0.0002 M	0.5	—0.35	—0.18
	2.1	—0.030	0.033		2.2	—0.60	—0.07
	7.8	0.011	0.047		7.2	—1.335	—0.27
	25.3	0.084	0.093		24.8	—1.73	—0.10
	50.1	0.22	0.173		49.3	—1.2	0.25
	74.4	0.35	0.435		74.1	—0.92	>0.9
	92.5	0.42	0.54		92.0	—0.65	1.0 appr.

2. *Potassium bromide.*

Three concentrations of this salt were employed, namely, 0.02 M, 0.005 M, and 0.001 M. The standard technique was used. It has already been mentioned that the bromide in the samples taken from the solutions of initial concentration 0.02 M was determined by titration using an adsorption indicator, while the course of bromide absorption from the more dilute solutions was followed by polarographic analysis. It has also been pointed out that the polarograph does not distinguish sufficiently between chloride and bromide, so that any exosmosis of chloride from the tissue will result in the bromide determinations in the experiments with 0.005 M and 0.001 M being too high, so that the absorption values will be too low. The results for the absorption of potassium bromide, given in Table VI, are to be regarded as minimum values of absorption, at any rate as far as the bromide is concerned. The error arising from this source is, however, judging from the results with potassium chloride, not likely to be appreciable. The results are recorded graphically in Figs. 3 and 4. A comparison of the results with those obtained with potassium chloride indicate a close similarity between the two, both as regards the general course of absorption and the rates of absorption of the ions in the two cases.

TABLE VI

*Course of Absorption of Potassium Bromide by Carrot Root Tissue*

Initial concentration.	Time (hrs.).	Absorption	
		K.	Br.
0.02 M	5.33	—0.01	0.03
	18.9	0.08	0.115
	28.7	0.15	0.19
	51.8	0.215	0.26
	73.75	0.24	0.30
	93.9	0.30	0.36
0.005 M	0.67	0.07	—0.023
	6.0	0.145	0.24
	19.6	0.34	0.49
	29.2	0.46	0.54
	52.4	0.59	0.59
	74.25	0.64	0.76
	94.4	0.71	0.86
0.001 M	0.67	0.07	0.00
	6.0	0.12	0.19
	19.7	0.38	0.49
	29.4	0.51	0.76
	52.6	0.57	0.82
	74.4	0.56	0.84
	94.6	0.69	0.91

With the two weaker solutions most of the bromide is absorbed by the tissue in four days, and the absorption of the anion, as in the case of potassium

chloride, exceeds that of the kation. The latter is also the case with absorption from 0.02 M potassium bromide, but the difference in the absorption of the two ions is here not very great.

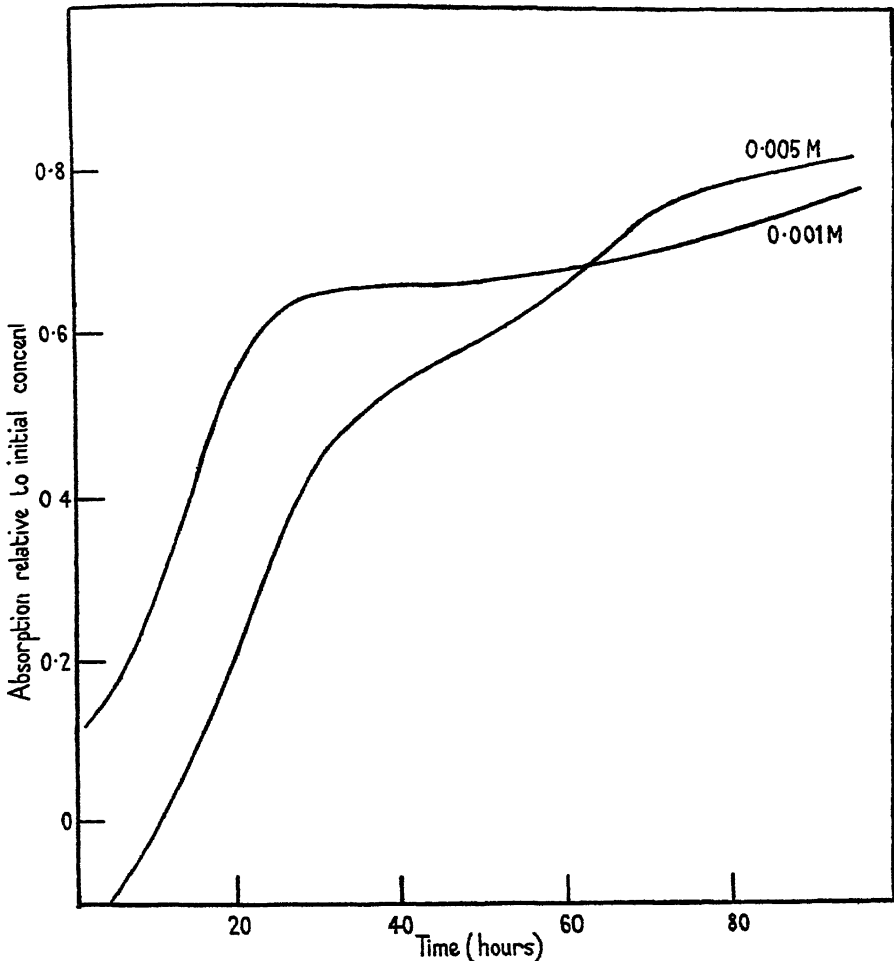


FIG. 5. Curves showing the course of absorption of potassium by carrot tissue from solutions of potassium nitrate having initial concentrations of 0.005 M, and 0.001 M.

### 3. Potassium nitrate.

The absorption of potassium nitrate by carrot tissue was followed in the same way as that of potassium chloride and potassium bromide. The same three concentrations were used and the course of absorption of the ions followed in every case, with the exception of the 0.02 M solution. Here the course of nitrate absorption was followed, but only the final value of potassium



absorption was determined. The results obtained are summarized in Table VII and shown graphically in Figs. 5 and 6.

The absorption of potassium nitrate follows a very similar course to that of potassium chloride and potassium bromide. As with these salts the absorption

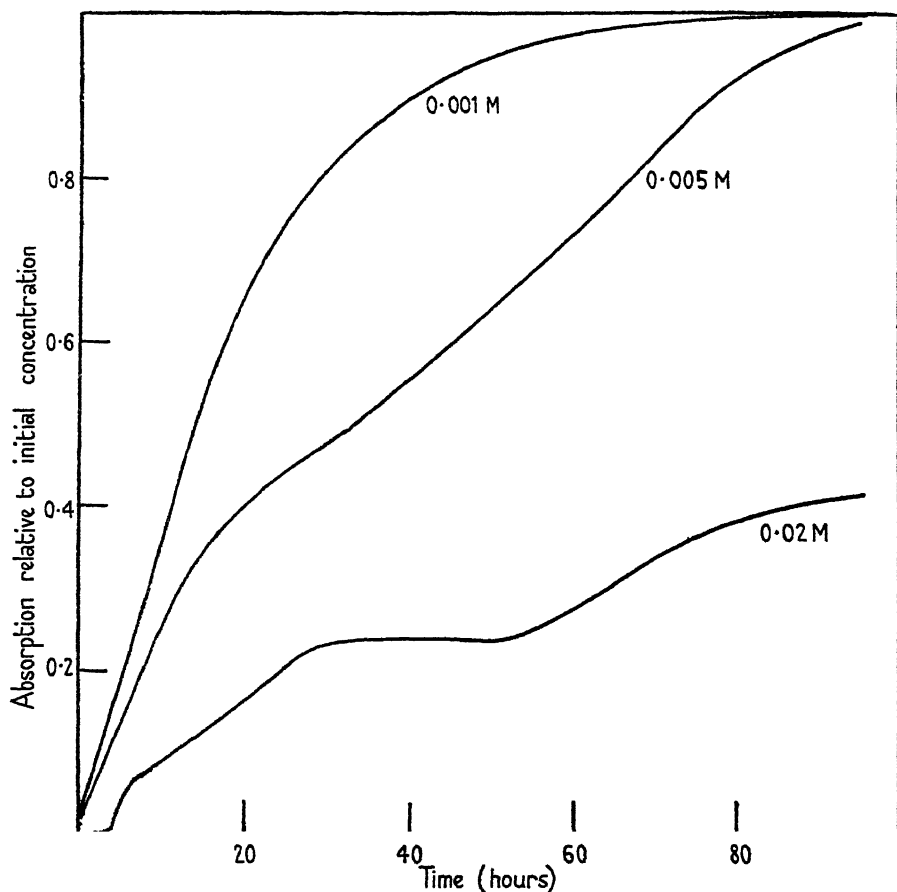


FIG. 6. Curves showing the course of absorption of nitrate by carrot tissue from solutions of potassium nitrate having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

of the anion exceeds that of the kation except with the strongest solution used, while by the end of the experiment practically all the anion in the solution has been removed by the tissue. Indeed, the absorption of nitrate is more rapid than that of chloride or bromide in every concentration, the whole of the nitrate in the 0.001 M solution having been absorbed in little more than two days.

#### 4. Potassium dihydrogen phosphate.

The same three concentrations of this salt were used, namely 0.02 M, 0.005 M, and 0.001 M. Owing possibly to the presence of free hydrogen ions

in the solution, the polarographic determination of potassium is not so straightforward as with the other salts used, and the results are in consequence less exact. As the results collected in Table VIII show, the absorption proceeds similarly to that of the salts already dealt with. These experiments were

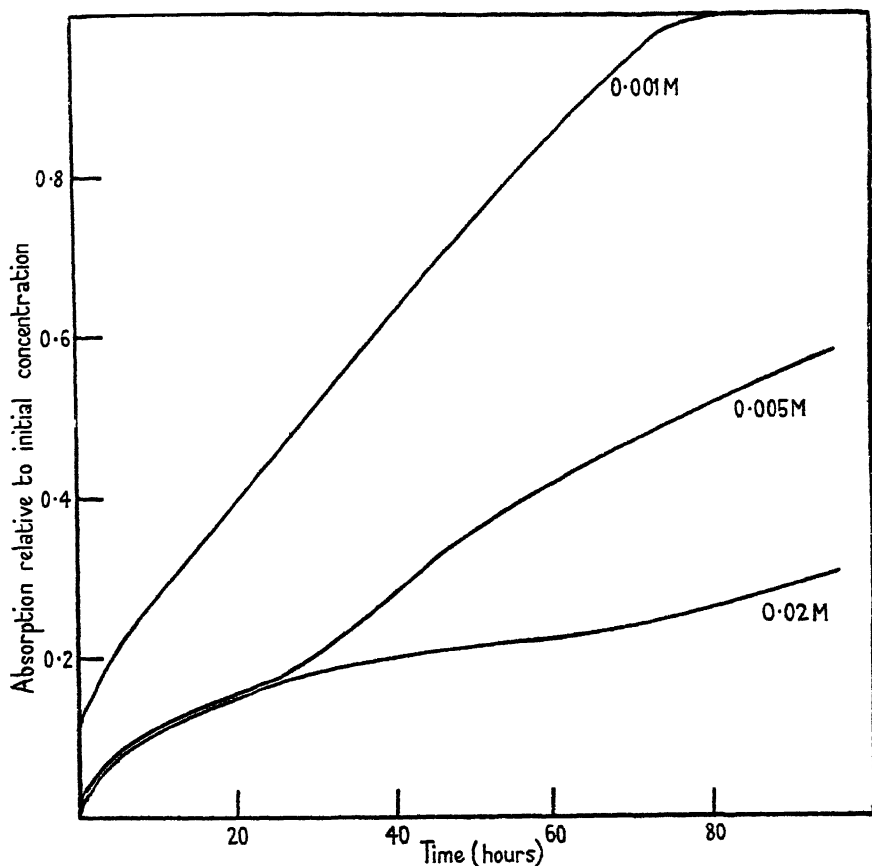


FIG. 7. Curves showing the course of absorption of phosphorus by carrot tissue from solutions of potassium dihydrogen phosphate having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

carried out in July with the new season's carrots, which had thus spent a negligible time in storage. The initial exosmosis of potassium was much greater than in the previous cases recorded, but it cannot be said at present whether this is related to the age of the tissue, the acidity of the solution, or the nature of the anion. The absorption of phosphate exceeded that of potassium, and only a negligible amount of phosphorus was left in the weakest solution at the end of the experiment. The results are shown graphically in Fig. 7, from which it is evident that absorption follows a similar course to that of the salts already examined.

TABLE VII

*Course of Absorption of Potassium Nitrate by Carrot Root Tissue*

Initial concentration.	Time (hrs.).	Absorption	
		K.	NO <sub>3</sub> .
0.02 M	6.0	—	0.06
	19.7	—	0.155
	29.3	—	0.24
	52.5	—	0.235
	74.3	—	0.36
	95.6	0.42	0.42
0.005 M	0.6	—0.13	0.03
	6.0	—0.06	0.15
	19.7	0.21	0.40
	29.3	0.40	0.47
	52.5	0.61	0.67
	74.3	0.77	0.92
	95.6	0.82	>0.95
0.001 M	0.6	0.12	—0.07
	6.0	0.18	0.21
	19.7	0.56	0.74
	29.3	0.65	0.95
	52.5	0.67	>0.95
	74.3	0.71	>0.95
	95.6	0.78	>0.95

TABLE VIII

*Absorption of Potassium Dihydrogen Phosphate by Carrot Root Tissue*

Initial concentration.	Time (hrs.).	Absorption	
		Potassium.	Phosphorus.
0.02 M	1.2	0.033	0.033
	7.7	—	0.091
	27.1	0.087	0.179
	52.0	0.095	0.218
	76.4	0.237	0.255
	96.3	0.297	0.313
0.005 M	1.1	—0.068	0.007
	7.6	—0.010	0.099
	27.2	0.156	0.185
	51.75	0.1845	0.373
	76.25	0.486	0.497
	96.2	0.554	0.577
0.001 M	1.0	—1.416	0.132
	7.3	—1.291	0.241
	26.9	—0.797	0.476
	51.5	—0.591	0.759
	76.0	0.159	>0.97
	96.0	0.736	1.0 approx.

5. *Potassium sulphate.*

The concentrations used of this salt were again 0.02 M, 0.005 M, and 0.001 M. The results of experiments carried out in July with the new season's roots are shown in Table IX, and graphically in Figs. 8 and 9.

TABLE IX

*Absorption of Potassium Sulphate by Carrot Root Tissue*

Initial concentration.	Time (hrs.).	Absorption	
		K.	SO <sub>4</sub> .
0.02 M	0.92	—0.062	0.056
	5.5	0.022	0.061
	27.0	0.034	0.066
	49.6	0.088	0.088
	73.8	0.101	0.116
	94.0	0.126	0.136
0.005 M	0.83	—0.032	0.054
	5.25	—0.038	0.061
	27.0	—0.044	—
	49.5	—0.0425	0.114
	73.8	0.089	0.164
	93.8	0.179	0.202
0.001 M	0.83	—0.078	—
	5.6	—0.050	0.009
	27.1	—0.013	0.041
	49.75	0.129	0.096
	73.9	0.174	0.214
	94.0	0.315	0.268

The outstanding feature of the absorption of potassium sulphate is the much lower rate of absorption of the ions of this salt as compared with that of the other potassium salts used. However, as with these other salts, in all these determinations, with only one or two exceptions on which obviously too much stress must not be laid, the absorption of anion exceeds that of the kation.

In a second series of experiments, carried out in June with old carrots at the end of the storage season, no absorption of potassium could be detected over a four-day period even from the lowest concentration of the salt, while sulphate was removed only to the extent of a few per cent. There is thus the possibility that with increasing age of the storage tissue the capacity for salt absorption declines.

## DISCUSSION

1. *The course of absorption.*

A review of the results collected in Tables III–IX and in Figs. 1–9 indicates that while the course of absorption shows certain variations in relation to the salt and its concentration, there is, nevertheless, a definite

uniformity throughout. In the case of potassium there is initially a tendency to exosmosis of this kation from the tissue. This is most obvious in the weakest concentrations, where the net result of the tendency to exosmosis and tendency to absorption is exosmosis, so that the external solution increases in

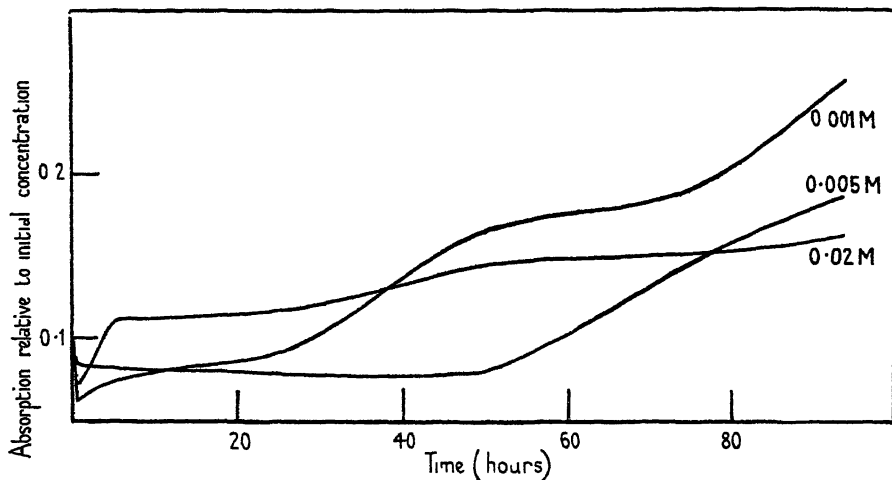


FIG. 8. Curves showing the course of absorption of potassium by carrot tissue from solutions of potassium sulphate having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

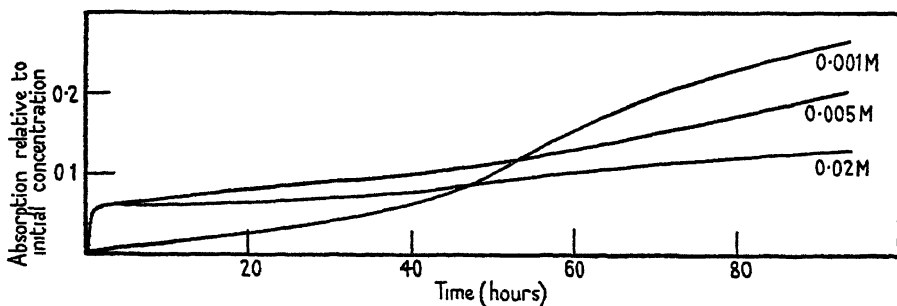


FIG. 9. Curves showing the course of absorption of sulphate from solutions of potassium sulphate having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

potassium concentration. In higher concentrations the tendency to absorption is usually greater than that to exosmosis, and the result is only a depression in the rate of absorption as compared with that of the anion. This would suggest that the exosmosis is unaffected, or little affected, by the presence of the salt in the external solution, and that it is, indeed, the same phenomenon as that exhibited by storage tissue when placed in distilled water (Stiles, 1927). On the other hand, the exosmosis was much greater in the case of potassium dihydrogen phosphate than in that of other salts examined. This might be

due either to some unusual factor of the particular batch of tissue examined, or to the specific action of this salt, such as might result, for example, from the presence of free hydrogen ions in the solution. That exosmosis is brought about by acids is a well-established fact (cf. Hind, 1916; Stiles and Rees, 1935).

Sooner or later the exosmosis of potassium gives place to absorption, this change occurring the sooner the more concentrated the external solution. It may again be noted that the rate of exosmosis into distilled water rapidly falls off, and that after a time the exuded electrolytes are reabsorbed.

For a time, usually about two or three days, the rate of absorption, rapid at first, gradually declines. This course does not, however, continue, and gives place to a phase during which the rate of absorption increases. This is the more noteworthy, since by the time this occurs the concentration of the external solution may have fallen considerably, often to half or less of its original value. This more rapid rate of absorption may continue until by the end of the experiment, usually after four days from the beginning, practically the whole of the ion may have been absorbed from the solution.

The course of anion absorption is similar, except that there is generally no appreciable exosmosis when the external solution is in a concentration of 0.001 M or higher. There was, however, a very definite exosmosis of anion when the external solution was 0.0002 M potassium chloride, although obviously this exosmosis is less than that of potassium. Thus, in general, only the two other phases are obvious, an initial phase during which the rate of intake decreases as if absorption was proceeding to an equilibrium, followed by a phase of increased rate of absorption. The latter proceeds so rapidly that in several experiments no appreciable amount of anion was left in the external solution by the end of four days; indeed, practically all the nitrate from a solution of initial concentration of 0.001 M was absorbed after 29 hours in the solution. There must be, of course, a more or less rapid falling off in the rate of absorption of any ion as the concentration of the ion in the external solution approaches zero.

Only two of the time-absorption curves obtained in this work fail to show this two-phase or three-phase course. These are the curves of anion absorption from 0.001 M potassium nitrate and 0.001 M potassium dihydrogen phosphate, and even here the divergence from the usual course is only apparent. The absorption of  $\text{NO}_3$  from 0.001 M potassium nitrate is so rapid that by the time the increased rate of absorption usually occurs there is practically no  $\text{NO}_3$  left in the external solution. The absorption of phosphate from 0.001 M potassium dihydrogen phosphate, although less rapid than that of  $\text{NO}_3$  from 0.001 M, is still very rapid, and although the external concentration is falling rapidly, the rate of absorption remains practically constant until its final fall due to exhaustion of the external solution, thus indicating that here the tendency to increased absorption is simply masked by the falling concentration of the external solution.

Since this course of absorption has not been recorded before, it is necessary

to re-examine the results previously obtained in experiments of this kind in the light of the present work. These previous results have already been noted and listed in Table I. It will be observed that the majority of these concern relatively short-period experiments lasting only one or two days, and so the phase of increased rate of absorption is not reached. This explains why the

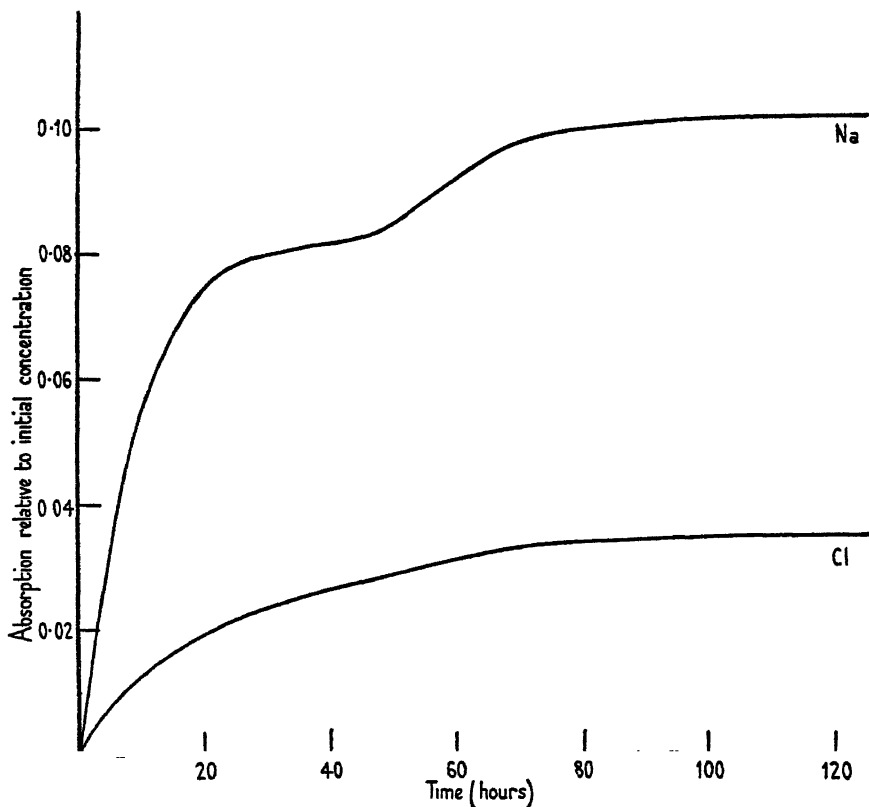


FIG. 10. Curves showing the course of absorption of sodium and chloride from a solution of sodium chloride having an initial concentration of 0.1 M. (Reconstructed from the data of Stiles, 1924.)

time-absorption curves in these experiments suggest that absorption is proceeding towards an equilibrium. Of the four remaining experiments, one dealt with the absorption by carrot of sodium chloride in a concentration, 0.1 M, very much higher than any used in this work. Nevertheless, when the results of this experiment (Stiles, 1924, Table VII, p. 626) are shown in graphical form as in Fig. 10, it will be observed that the time-absorption curves for both ions show the same two phases that characterize the results recorded in this paper, the increased rate of absorption again developing at about the end of the second day. There is, however, this difference, that the increased rate

of absorption is not long continued and falls off to a low value by the end of the experiment. Whether this difference is related to the high concentration of the salt or to a diminution in oxygen supply as a result of the long period (two days) over which the supply was not renewed cannot be stated; it is conceivable that both factors operated.

The second of these earlier long-period experiments concerned the absorption of potassium bromide by potato. In this experiment Steward measured the absorption of both ions on four successive days. At the end of the experiment there was complete absorption of the bromide from the external solution. The initial phase of potassium exosmosis was very marked, as would be expected with the dilute solution used, namely, 0.000539 N. There was, however, no indication of increased rate of absorption after three or four days. This may be related to the tissue used having different properties from carrot; but having regard to the low concentration of the original solution, one would not expect to observe the phase of increased absorptive activity owing to the lowering of the concentration of the external solution. The course of absorption would, indeed, be similar to that recorded for nitrate and dihydrogen phosphate in low concentration.

The third of the earlier long-period experiments is that by Steward and Berry in which the absorption from potassium bromide by artichoke tuber of only the bromide was observed by daily measurements of the absorption over a six-day period. Here the concentration of the external solution was maintained at something approaching constancy by the daily replacement of the solution. There was, however, no indication of an increased rate of absorption at any time, the rate remaining approximately constant throughout.

The last of these earlier experiments is the recent one of Steward and Harrison in which the absorption by potato of both ions of rubidium bromide from a 0.002 N solution was measured on four successive days. Steward and Harrison claim that the rubidium ion was rapidly absorbed for a day or two, during which time the bromide ion was scarcely absorbed at all. After this time both ions were absorbed at a constant and the same rate, so that, in effect, absorption followed a two-phase course, a phase in which the rubidium ion was alone absorbed and presumably exchanged with potassium or other kation of the tissue, being followed by one in which the two ions entered in equivalent proportions. Actually, the results of Steward and Harrison's experiment scarcely justify this conclusion. In Fig. 11 the data published by these writers have been replotted and it will be observed that the relationship between time and absorption as given by the actual data can only be regarded as linear if the possible error of the determinations is considerably greater than we are given to suppose. And if this is so, there is no more reason to assume that the four or five points in question ought to lie on a straight line than on some other curve. But if we accept the determinations of Steward and Harrison as reasonably correct, what their results do indicate is that there is



an increased rate of absorption of both rubidium and bromide after about three days, and their results in this respect thus fall well into line with those recorded in this paper. Although with the scanty data provided by Steward and Harrison there is hardly sufficient warrant to assume that this is the

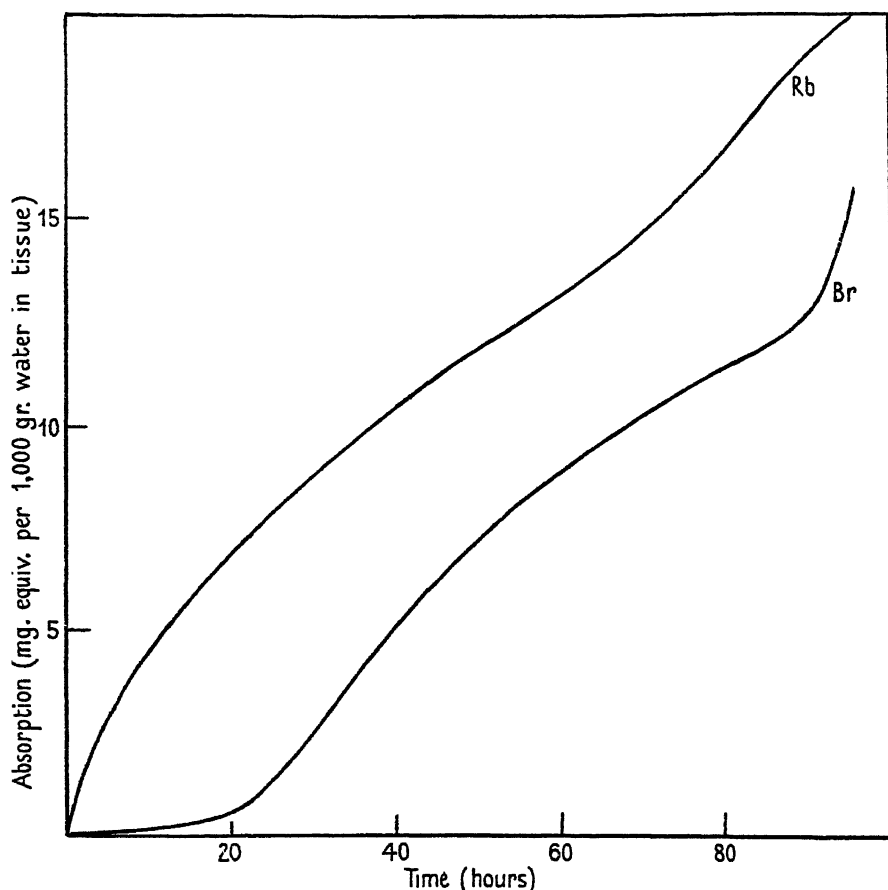


FIG. 11. Curves showing the course of absorption of rubidium and bromide from a solution of rubidium bromide having an initial concentration of 0.002 M. (Reconstructed from the data of Steward and Harrison, 1939.)

correct explanation of their results, there is as much justification for it as for their own interpretation of them.

The observed absorption of bromide by different tissues affords an interesting comparison. With carrot we have shown that the bromide ion is rapidly absorbed immediately the tissue is placed in a solution of potassium bromide, whereas Steward recorded only a slight absorption of this ion from potassium bromide by potato after 24 hours, while a similar behaviour was recorded by

Steward and Harrison for the absorption of bromide from rubidium bromide by this tissue. Artichoke, judging from the results of the experiment by Steward and Berry, appears to occupy an intermediate position. It would thus appear that different storage tissues may show different behaviour in regard to the absorption of an ion.

The two- or three-phase course in the absorption of the ions of potassium salts is a reflection of the complexity of the salt relations of plant tissue. The initial phase of exosmosis of potassium into dilute potassium salt solutions, followed by reabsorption, is similar to the phenomenon recorded in 1927 by one of us of the exosmosis of electrolytes in general into distilled water, followed by their reabsorption. A possible explanation of this then put forward was that the exosmosis was from dead and dying cells in the neighbourhood of the cut surface, diffusion being much more rapid into the external liquid than into the interior of the tissue. The comparatively slow absorption by the living cells continues all the time, so that as the dead or moribund tissue becomes exhausted of electrolytic contents and exosmosis draws to an end the observed exosmosis gives place to absorption from the external solution. This explanation would hold equally well for potassium salts in particular as for electrolytes in general, provided the concentration of the salt is lower in the external solution than in the dead cells. It is true this view has not found favour with other workers in this field, but no sound argument or evidence has been produced against it. Briggs and Petrie, for example, state that there is no direct evidence in favour of this explanation, but their argument, if so it is intended, for this statement, that 'the tissue, small in bulk relative to the solution, would have to absorb the greater part of the electrolytes liberated' and that, as far as we know, tissue such as that used, 'never decreases the conductivity of such dilute solutions, except in experiments of the type we are trying to explain', eludes us. The fact remains that electrolytes do pass out into the external medium, and can be reabsorbed from the resulting dilute solution so that the electrical conductivity is reduced to a very low value.

Briggs and Petrie themselves emphasize the importance of respiration in the interchange of ions between a tissue and an external solution. Respiration produces the two ions of carbonic acid,  $H$  and  $HCO_3$ , in the tissues, and if the  $H$  ion has a greater mobility than the  $HCO_3$  ion, the external solution will tend to be positive to the tissue, and if the former contains potassium, this will tend to be in greater concentration in the tissue than in the external solution, while the reverse will be the case for an anion. Hence an increase in respiration will emphasize this condition, so that it will bring about an intake of potassium ions and a loss of anions. On the other hand, there are in the tissues substances with non-diffusible ions, such as proteins, the degree of ionization of which depends on the hydrogen-ion concentration. An increase in the rate of respiration, by increasing the hydrogen-ion concentration, will bring about a depression in the degree of ionization of acidic electrolytes—those with non-diffusible anions—and an increase in the degree of ionization

of basic electrolytes—those possessing non-diffusible kations. As a result diffusible kations will be lost from the tissue and diffusible anions will be absorbed.

As far, then, as respiration is concerned, the absorption or excretion of potassium would be determined by a change in the respiration rate and the effect of this on the ionization of cell constituents with immobile ions together with a difference in mobility of H and  $\text{HCO}_3$  in the tissue. Certainly when the external liquid is originally distilled water, the observations of Briggs and Petrie indicate that the phase of exosmosis corresponds with one of increasing respiration rate, while the phase of absorption corresponds with one of decreasing respiration rate. But it is difficult to see how the effects of respiration emphasized by Briggs and Petrie can have much bearing on the results described in this paper, for the following reasons. In the first place the amount of the initial exosmosis of potassium into a solution of a potassium salt, or whether it occurs at all, depends on the concentration of the salt. With higher concentrations there is generally an absorption of potassium ions from the beginning, while the exosmosis relative to the concentration is more marked the lower the concentration. Secondly, there is no evidence that continued absorption of potassium is generally accompanied by progressive reduction in the respiration rate; indeed, Steward and his collaborators have urged the contrary, namely, that a high respiration rate is associated with a high rate of absorption. Thirdly, the scheme of Briggs and Petrie requires that along with absorption of kation there should be exosmosis of anion, and vice versa. Inspection of the tables in this paper will show, however, that during the initial period of an experiment there may be absorption of both ions, absorption of one and exosmosis of the other, or exosmosis of both, as in the case of 0.0002 N potassium chloride.

Hence, while not disputing the possible effects of respiration on the interchange of ions between cell and surroundings, we feel that the phase of exosmosis of potassium observed in these experiments is more readily explained on the lines already propounded by one of us than in terms of the effect of respiration. We readily admit, however, that other effects may be operative.

Apart from any preliminary exosmosis, and with the few exceptions already noted, the absorption of all the ions examined follows a two-phase course, the first phase being one of comparatively rapid initial absorption with regular progressive decline in rate, which is followed by a second phase marked by an increase in the rate of absorption which falls off finally owing, at least in some cases, to exhaustion of the external medium in respect of the ion in question. The simplest explanation of this behaviour is that the effect of immersing the tissue in a solution of one of the salts examined is to bring about an increase in the permeability of the cell membranes so that the rate of diffusion of the salt into the cells of the tissue is increased. Such an effect of monovalent ions has frequently been suggested (cf. Osterhout, 1912 *a*, *b*

Brooks, 1916; Asprey, 1935). However, one would expect that if this were so the increased rate of intake would occur the sooner the higher the concentration of salt, but there is no evidence of this, the increase being observable at about the same time in all concentrations. However, more precise information is desirable before dismissing this explanation. If the increased rate of absorption is not related to the action of the salt itself it must be due to some change in the tissues with time independent to a great extent of the medium in which it is immersed, and involving increased cell activity of some kind, such as would occur if rejuvenescence of some cells of the tissues occurred following on the wounding to which the tissue is subjected by its preparation in the form of discs. In this connexion we may note the conclusions reached by Berry and Steward (1934), based on a comparison of bromide absorption by a number of different kinds of tissue, that 'the capacity to accumulate salt rapidly from dilute solution is essentially a property of cells, no longer strictly "storage" or "resting" cells, but actually much more active than the mere absence of external signs of growth would indicate', and indeed, undergoing vital activities leading to cell division.

If this latter view of the matter is accepted the form of the time-absorption curves indicates that absorption of any of the ions examined involves two processes: an initial absorption of the ion towards a position of equilibrium, followed, or probably overlapped, by a phase of increasing absorptive activity conditioned by the increasing metabolic or growth activity of the tissue. The view that absorption consists of two processes has already been developed by Steward (1935), who would distinguish between 'primary salt absorption' which occurs in actively growing metabolizing systems and which involves accumulation of both cations and anions, and 'induced absorption' which occurs with relatively mature cells and which consists of an interchange of ions with the external medium without change in the ionic concentration of the latter.

In analysing the results of their experiment on the absorption of rubidium bromide Steward and Harrison interpret the course of uptake of the ions of this salt as indicating a first phase of induced absorption of rubidium unaccompanied by bromide, which is followed by prolonged primary absorption of both ions in equivalent amounts. The induced absorption could be compared with adsorption, but the subsequent primary absorption could not.

From what has been written earlier it will be clear that we are broadly in agreement with Steward and Harrison as to the existence of two phases in salt absorption, and it is at least possible that the two phases may correspond with the two types of absorption hypothesized by Steward. We further agree that the earlier shorter period experiments in which there appeared to be an approach to an equilibrium, and in which the relation between concentration and absorption approximates to the adsorption equation, are concerned with the first phase of absorption.

We do not, however consider that in the present state of our knowledge it would be profitable further to discuss whether the two phases in the time-absorption curves result from two independent absorption processes such as hypothesized by Steward or whether they result from a single process modified by a change in cell permeability. It is hoped to produce more information bearing on this point later.

## 2. *The unequal absorption of ions.*

There is no need to emphasize the universality of the phenomenon of unequal absorption of the ions of a salt in experiments of the type here described, nor do we at this stage propose to discuss further the part played by adsorption and by the Donnan equilibrium in bringing about this inequality of absorption. We may, however, examine the justification of the claim of Steward and Harrison (1939) that the two ions of rubidium bromide are unequally absorbed only during the first phase of absorption. In the following table are shown the quantities of the two ions of rubidium bromide absorbed during successive periods as given by Steward and Harrison in their Table II. It must surely be admitted that there is as little evidence from these data of equality of absorption of the two ions during any stage of the absorption, as there is for the other conclusion drawn by Steward and Harrison from these data, namely, that after a preliminary period the rate of absorption of both ions is constant.

TABLE X

### *Absorption of the Ions of Rubidium Bromide during Successive Periods of Time*

(Data from Steward and Harrison)

Period.	Duration of period.	Absorption		Percentage excess of more absorbed ion.
		Rb.	Br.	
1	21.7	7.2	0.70	—
2	24.3	4.2	5.72	36
3	21.0	2.8	3.49	25
4	23.2	4.9	2.89	70
5	5.3	0.9	3.0	233

In general, our own experiments show as near an approach to equality of absorption of kation and anion as those of Steward and Harrison, but we cannot assume that the observed divergences from this equality are all due to experimental error. It must be realized that where differences in the rate of absorption of the two ions occur the balance of positive and negative ions is probably maintained by diffusion of ions from the tissue. The availability of ions for this must impose a limit on the extent to which inequality of ionic absorption is possible. Further, bearing this in mind, we should expect the

relative inequality of absorption to be more marked from weaker solutions. An inspection of Tables III to IX shows that this is actually so.

### 3. *The influence of concentration.*

The influence of concentration on the rate of absorption has frequently been discussed since this was first emphasized by Stiles and Kidd twenty years ago. The experiments then carried out were for the most part short experiments of about two days' duration, and the time-absorption curves then obtained suggested that absorption proceeded towards a position of equilibrium. As already pointed out the experiments described in the present paper show that the first phase suggesting absorption to an equilibrium is followed by a phase of increased absorption, so that, in fact, a position of equilibrium in uptake is not reached during the course of experiments lasting four or five days, except in so far as such is conditioned by exhaustion of the external solution.

The results obtained in the work described in this paper indicate, however, that the effect of concentration is much the same for both phases of absorption, namely, the rate of absorption relative to the concentration of the external solution is the greater the lower the concentration. While obviously the conception of an absorption ratio of final internal to final external concentration at *equilibrium* can no longer be held, we can still retain the term absorption ratio for the ratio of internal to external concentration at any time during absorption. It is clear that in all the cases we have examined the absorption ratios of the ions of all the salts we have used can reach values many times unity in all the concentrations employed, and that, allowing for the presence of the ion initially in the tissue, the value of the absorption ratio increases with decrease in concentration. For a further analysis of the absorptive process and the effect of concentration, considerably more experimental data are necessary and a further discussion of the matter at this stage would not be profitable.

## SUMMARY

1. The absorption by carrot root tissue of both ions of a number of potassium salts, namely the chloride, bromide, nitrate, dihydrogen phosphate, and sulphate, has been followed over a period of four or five days, by determining the fall in concentration of the external solution. For the determinations use has been made of adsorption indicators, the polarograph and the Spekker absorptiometer, by which small quantities can be measured with a reasonable degree of accuracy.

2. From all salts except sulphate absorption of both ions proceeds rapidly, although the absorption of potassium may be preceded by a period of ex-osmosis from the tissue. This potassium is, however, always reabsorbed. The rate of intake of the ions of potassium sulphate, although notably less than

those of the other salts examined, is nevertheless quite definite in younger carrots. At the end of the storage season it may, however, be negligible.

3. Apart from any initial exosmosis the absorption of all ions examined follows a two-phase course. The first phase is characterized by more rapid initial absorption, which falls off with time. The second phase, which becomes evident after two or three days, is characterized by an increased rate of absorption. The falling off in this rate is conditioned by exhaustion of the external solution in respect of the ion concerned. In the few cases where the increased rate is not observed the failure is due to the same reason, namely, the lowering of the concentration of the external solution.

4. The two-phase course of absorption can be explained as due either to an increase in permeability of cell membranes, as a result of exposure to a solution of the salt employed, or to absorption being of two kinds as hypothesized by Steward. It is held that evidence at present is inadequate to decide which of these views is correct.

5. The two ions of a salt are absorbed to unequal extents, but as such unequal absorption involves exosmosis of ions from the tissue, the degree of unequal absorption depends on the extent to which exosmosis of ions is possible. Hence the departure from equality in the rate of absorption of kations and anions is more marked with dilute than with stronger solutions, since the concentration of ions available for exosmosis will be relatively less in relation to the quantities of ions absorbed the higher the concentration of the external solution.

The work described in this paper was rendered possible by grants from the Research Committee of the University of Birmingham, and the Government Grants Committee of the Royal Society. To both these bodies we would here record our thanks for their assistance.

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# An Experimental Study of the Permeability to Gases of the Seed-coat Membranes of *Cucurbita Pepo*

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With one Figure in the Text

## INTRODUCTION

**D**URING the course of certain investigations conducted in this laboratory on the conditions that control the germination rate, data were obtained which suggested that one of the dominant factors involved was the rate at which gases traverse the membranes of the seed-coat. For a full analysis of the relation between the permeability of the seed-coat to gases and the germination rate, data on the absolute rates at which gases traverse these membranes are clearly necessary. The subject has been examined by a number of investigators, but their observations yield relative, not absolute, rates of permeation. The present investigation was therefore undertaken with the purpose of measuring the absolute rates at which gases traverse the membrane. It was soon found that the properties of the membranes of the seed-coat may vary considerably, and the investigation was therefore extended to cover the changes in the permeability of the membranes which can be induced by various treatments.

The first observations on the permeability to gases of seed-coat membranes were made by Wiesner and Molisch (1890). These workers published the results of a comprehensive investigation of the movement of gases across a variety of plant membranes. Among other membranes they used the seed-coats of peas and beans. They found that gas could not readily be forced through these membranes even by a relatively high pressure. This observation was later confirmed by Becquerel (1907). Wiesner and Molisch (1890) also found that the dry membrane was always relatively less permeable to diffusion of gases than the wet; and that carbon dioxide diffused through these membranes relatively more rapidly than did either nitrogen or oxygen. Crocker (1906) in the course of an investigation on the causes of the difference in the rates of the germination of the upper and lower seeds in the burr of *Xanthium* found it necessary to examine the rates of diffusion of oxygen through the wet and dry seed-coats of this plant. He reported that the dry was always relatively more permeable to gases than the wet membrane. Shull repeated the work of Crocker, but could not confirm his results; he ascribed Crocker's finding to the probable use of dry membranes which were imperfect.

Kidd and West (1917) studied the permeabilities to carbon dioxide and

oxygen of seed-coats of *Brassica alba* which had been pre-treated with CO<sub>2</sub>. Unfortunately the rates of diffusion of oxygen and carbon dioxide were determined by methods with which the area maintaining the process could not be defined. The rates of diffusion of the two gases cannot therefore be compared. The method employed of measuring the relative rates of diffusion of oxygen is, however, of some interest and will be described in a later section.

#### MATERIALS AND METHOD

The membranes of the seed-coat of *Cucurbita Pepo* are eminently suitable for the purpose of the present investigation, since they are large and can be easily separated from the rest of the seed. The seed-coat of this species is not a single morphological unit but consists of two parts each of which can be removed separately. There is an outer thick rigid and an inner much thinner and more pliable membrane. These are separated by an air-gap which is traversed by groups of dead thin-walled cells.

The outer membrane is readily removed from either the wet or the dry seed. The technique adopted in this investigation was that of cutting along the flanks of the seed with sharp dissecting scissors, and then of lifting the isolated halves with a scalpel inserted into the air-gap between the two membranes. The inner membrane when dry is closely adherent to the surfaces of the cotyledons and the embryo and can be separated from these only after soaking the seed in water for about an hour. Thus whether wet or dry membranes were required for experimental purposes, all were prepared from soaked seeds. It was found easier to remove the inner membranes from the separated cotyledons than from the whole seed. After removal of the outer membranes the embryo was cut away. This operation exposed the line of contact of the two cotyledons, and between these a scalpel could now be inserted; the cutting edge of the scalpel was then worked round the periphery of the seed, thus severing completely the membrane enclosing the cotyledons. As this membrane projects some distance beyond the apex of the cotyledon, the projecting strip of the membrane could at this stage be gripped firmly with a pair of forceps and the whole then pulled away from the cotyledons. If the operation is carefully performed, no damage to the membrane results.

If the inner membrane is required in the wet condition, it can be used immediately after separation from the seed. If, on the other hand, it is required in the dry condition, then the membrane has to be exposed for some time to a dry atmosphere before use; the drying, however, must be done with some care. In the dry state the membrane is brittle, and unless it lies flat over the surface against which it is clamped in the apparatus it may develop cracks. It is therefore necessary to ensure that during drying the membrane does not wrinkle, and this was done by flattening it against a glass sheet with a rubber roller, and by allowing it to dry in that position. Under the standard conditions used, exposure to the atmosphere for six hours was found sufficient to dry the membrane.

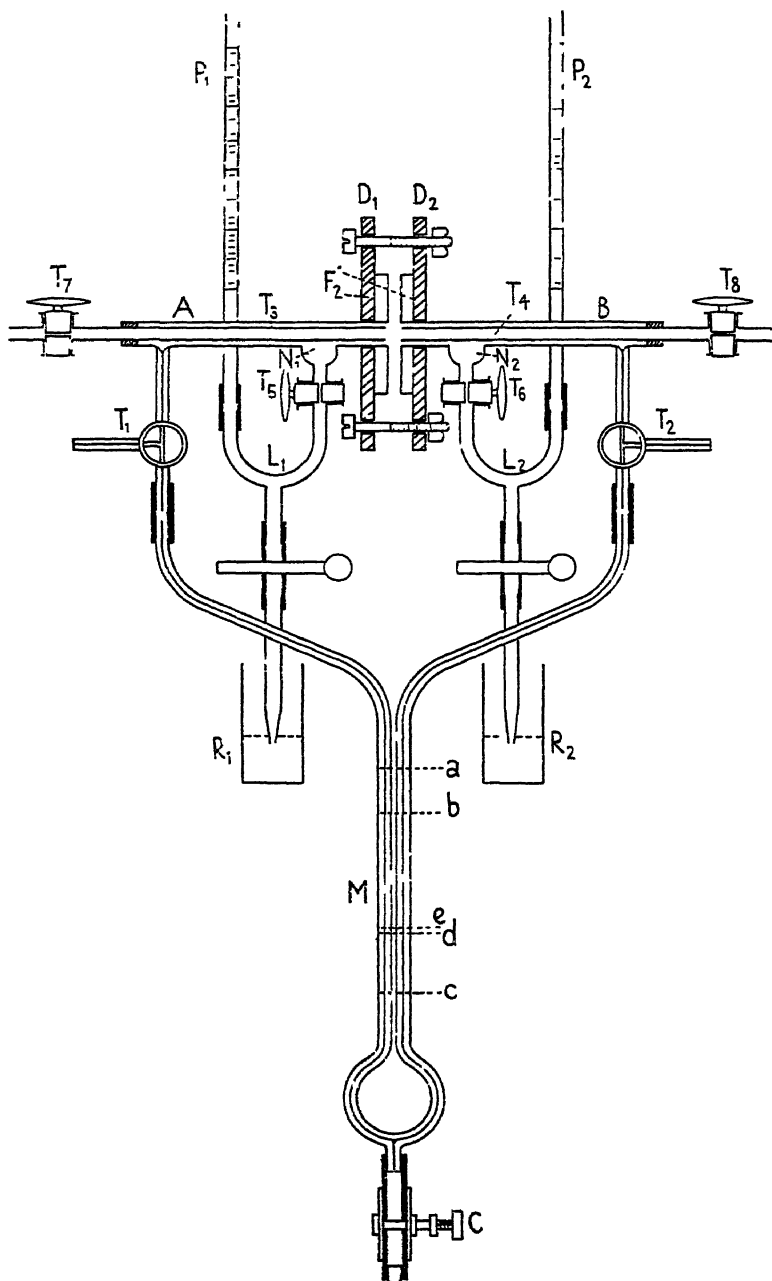
## APPARATUS

The apparatus devised for the purposes of the present investigation differs very considerably from that used by earlier workers. Wiesner and Molisch (1890) observed the change in height of a column of mercury which was supported in a tube which formed the long and perpendicular arm of a T. One end of the horizontal part of the T was closed by a clip on a length of rubber tubing, and the other by the membrane whose permeability was being measured, the space over the mercury being occupied by the gas studied. The apparatus used by Crocker (1906) and, later, by Shull (1913) was more elaborate. It consisted in principle of a horizontal glass tube, carrying a drop of indicator liquid, which is continued downwards as a vertical limb and which is closed by the experimental membrane. The space between the membrane and the drop of indicator is occupied by air at atmospheric pressure. The end of the tube carrying the membrane is enclosed in a chamber containing nitrogen, and the membrane is suspended above a surface of pyrogallol to act as oxygen absorber. Both with this apparatus and also with that of Wiesner and Molisch relative diffusion rates of different gases may be conveniently studied, but absolute rates cannot be so determined since the change in position of the indicator is due not to the diffusion rate of a single gas across the membrane but to the difference in the rates of diffusion of the gases on the two sides of the membrane.

Kidd and West (1917) measured the relative rates of diffusion of oxygen across various seed-coats of *Brassica alba* by observing the rise in level of sodium hydroxide in a tube which held oxygen, and the free end of which, closed by the experimental membrane, was placed in an atmosphere of carbon dioxide. The gas diffusing into the tube was immediately absorbed by the sodium hydroxide; the change in level in the tube was then due solely to the diffusion outward of oxygen.

The apparatus used in this investigation is shown in the figure. The membrane studied is inserted between two glass flanges  $F_1$ ,  $F_2$ , ground flat and carried at the ends of two tubes A and B. The two glass flanges are brought together by the pressure of two ebonite discs  $D_1$  and  $D_2$ , through which the glass tubes pass and which can be moved together by means of four brass screws provided with nuts. It is essential that these ebonite discs should register exactly, to which end they were cut from a single disc after it had been drilled to carry the screws and the tubes A and B. As already stated, the membrane is inserted between the glass flanges and held in position by pressure. In order to define the area of the membrane serving as diffusion path it is necessary that the ends of the tubes A and B should register exactly, which would not be possible if the tubes had rounded edges; to prevent this the flanges were sealed in the ends of the tubes with sealing wax and the whole ground flat with carborundum powder on a glass plate.

To ensure a gas-tight seal with the membrane without exerting a pressure



Apparatus used in the investigation of the permeability to gases of the seed-coat membrane.

sufficient to cause injury presents practical difficulties. After many trials a satisfactory luting material was found which consists of about equal weights of kaolin and pure lanolin thoroughly mixed into a stiff paste. This mixture was found by trial to be non-absorbent for all the gases studied and also to provide a gas-tight seal without necessitating undue pressure on the membrane; this material has the further advantage that it is non-absorbent for water and it does not cause infiltration of the membrane.

The tubes A and B are provided with side arms carrying three-way taps  $T_1$  and  $T_2$ . Wherever convenient capillary tubing was used in the construction of the apparatus in order to keep the internal volume as low as possible, thus increasing the sensitivity. The gases used in the experiment were released from cylinders, and introduced through the horizontal arms of the taps  $T_1$  and  $T_2$ , and were led out of the apparatus through the tubes  $T_3$ ,  $T_4$ , which traversed the length of the tubes A and B leaving narrow annular spaces. By this arrangement all dead spaces were avoided, and if necessary the gases could be swept out very rapidly. In order to prevent pressure being exerted on the membrane during the introduction of gases into the apparatus, the tubes  $T_3$ ,  $T_4$  were prepared from tubing whose internal diameter was considerably greater than that of the side arms of the three-way taps  $T_1$ ,  $T_2$ . The lower arms of these taps were attached with rubber tubing to two limbs of a manometer M, which was filled with Brodie's fluid. To the base of the manometer a side arm was fused carrying a thick-walled rubber tube closed at its lower end by a short length of glass rod. The rubber could be compressed between two metal plates by a screw clip c so that the level of the liquid in the manometer could be adjusted for the purpose described below.

The tubes A and B were also provided with two wide side arms  $N_1$ ,  $N_2$  which constituted two small cups to which taps  $T_5$ ,  $T_6$  were fused below leading to T-pieces  $L_1$ ,  $L_2$ ; the lower arms of these T-pieces carried rubber tubing provided with clips and tubes dipping into reservoirs  $R_1$ ,  $R_2$ . To the third arms of each of the T-pieces  $L_1$ ,  $L_2$  graduated pipettes  $P_1$ ,  $P_2$  of volume 1 c.c. were attached. The purpose of this arrangement was to provide means for introducing into the closed system caustic soda solution for the absorption of carbon dioxide. In order to do this the soda was sucked into the pipettes to a required level from the tubes  $R_1$ ,  $R_2$  and a measured volume was then released into the cups through the taps  $T_5$ ,  $T_6$ .

Different gases were used on the two sides of the membrane. Since carbon dioxide is easily absorbed, this was always used as one of the experimental pair of gases, the other side of the system being occupied by either oxygen or nitrogen. The two gases were introduced into the opposite sides of the apparatus at atmospheric pressure and the taps  $T_1$ ,  $T_2$ ,  $T_7$ ,  $T_8$  were then set to shut off completely all communication. Diffusion was allowed to proceed for a given period. During this time different volumes of the two gases traverse the membrane, and at the end of the experimental period there is a consequent increase of pressure on one side and a corresponding decrease on the other.

In this investigation pressures were not recorded, all measurements of change were made in terms of volume. The volume change was not measured on both sides but only on the side into which the carbon dioxide had penetrated. This was done by turning the three-way tap on the side into which carbon dioxide had penetrated so as to open communication with the manometer; on the other side the three-way tap was turned so that the manometer was here in communication with the atmosphere. By means of the screw clip *c* the fluid in the two arms of the manometer was brought to the same level. The difference between the final and the original levels was a measure of the volume change on the one side into which carbon dioxide had diffused.

The volume change on one side is a measure of the difference between the volumes of the two gases which have diffused in opposite directions. If now the total volume of one of the gases which has penetrated the membrane can be estimated, then the volume of the other gas which has diffused across the membrane can be calculated. Of the two gases  $\text{CO}_2$  is more convenient to estimate. On the side into which this gas had penetrated—i.e. the same side on which the net volume change had previously been measured—a known quantity of caustic soda was introduced into the apparatus. The arrangement of the three-way taps  $\tau_1$ ,  $\tau_2$  during this operation was such that on the side on which the caustic soda was introduced the manometer communicated with the apparatus, while on the other side it was open to the air. The introduction of the caustic soda caused an immediate depression of the fluid in the manometer. The pressure as recorded by the manometer, however, was continually changing owing to the absorption of the carbon dioxide. It was therefore not possible during the experiment to determine the depression of the level in the two arms after the whole system had been brought to atmospheric pressure due to the introduction of the standard volume of solution. The change in level could, however, be deduced from a previous calibration. After complete absorption of the carbon dioxide the liquid in the two arms of the manometer was again brought to the same level and now stood at a level higher than that following the introduction of the soda during the previous calibration. The difference between these two levels gave a measure of the total quantity of  $\text{CO}_2$  that had diffused across the membrane.

The absorption of the carbon dioxide just described occupied about fifteen minutes. During this time more of the gas, if present, would diffuse from the side into which it had originally been introduced at the beginning of the experiment. This was prevented by removing the carbon dioxide on this side and replacing it by either oxygen or nitrogen immediately after the soda had been introduced into the opposite side of the apparatus. The removal of the carbon dioxide and its complete replacement by another gas was effected within one minute.

When the removal of the carbon dioxide was complete from the side into which it had originally been introduced at the beginning of the experiment, this gas was now present only on that side of the membrane to which it had

diffused. At this stage the membrane separated a mixture of carbon dioxide and the second experimental gas, on the one side, and a pure sample of the second experimental gas on the other. During the period of absorption of carbon dioxide, diffusion of the second experimental gas into the side at the lower partial pressure would occur. This diffusion must necessarily increase the volume of gas in the system on this side and decrease the volume on the other by a corresponding amount; the side on which the volume increased being that into which carbon dioxide had diffused and was now being absorbed. The reduction in volume on this side after  $\text{CO}_2$  absorption as measured in the manometer was therefore not an absolutely accurate measure of the carbon dioxide present on that side. The accurate measure was obtained by adding to the volume change after absorption of  $\text{CO}_2$  indicated by the manometer reading, the reduction in volume on the opposite side. The value of this correction was determined by closing all connexions with the open air and the manometer on its opposite side after replacing the carbon dioxide which it originally contained by the second experimental gas. When the reduction in volume on the one side on which carbon dioxide had been absorbed had been measured, the arrangement of the three-way taps on the two sides was reversed. The manometer limb on the side on which carbon dioxide was absorbed was then open to the atmosphere through  $\tau_2$ , and the other limb then put in communication with the apparatus through  $\tau_1$ . The value of this correction was, however, small; it never exceeded 5 per cent. of the total measurement. It was in fact less than the difference between the values given by duplicate samples, and it can therefore be disregarded, as in fact it was in this investigation.

During the experimental period, and also when the  $\text{CO}_2$  was being absorbed, communication between the body of the apparatus and the manometer was always interrupted. This arrangement had a twofold purpose: (1) to prevent any possible leakage of the  $\text{CO}_2$  through the rubber connexions below  $\tau_1$  and  $\tau_2$ , and (2) to prevent any diffusion of water vapour upwards from the manometer into the body of the apparatus.

For the purpose of describing more precisely the sequence of operations that have been detailed above, a particular example in terms of the lettering of the figure is developed in the following paragraphs.

Suppose the side of B is occupied by  $\text{CO}_2$  and that of A by oxygen. At the end of the experimental period some  $\text{CO}_2$  will have penetrated into A. This volume change in A is alone measured. To do this at the end of the diffusion period tap  $\tau_1$  is turned into the position in which A is in communication with the manometer.  $\tau_2$  is turned so that the manometer on that side is in communication with the atmosphere. Before the measurement begins the level of the liquid in the two arms of the manometer stands at  $a$ . After side A has been put in communication with the manometer the fluid is displaced, due to the difference in volume of  $\text{CO}_2$  which has diffused into A and the oxygen which has diffused into B. The liquid in the two arms is then brought to the same



level *b*. This level is lower than that of *a*, and the difference between *a* and *b* is a measure of the net volume change on the side A.

The purpose of the next stage in the experiment is that of measuring the volume of diffused carbon dioxide. On side A a measured volume of soda is run through the tap  $T_5$ . This soda absorbs the diffused carbon dioxide, but during the time occupied by this process it is necessary to prevent further diffusion of  $\text{CO}_2$  from B. This is accomplished by circulating oxygen through side B, the gas being introduced through  $T_2$  and escaping through  $T_8$ ; this is continued for one minute, after which  $T_2$  and  $T_8$  are closed. When absorption of  $\text{CO}_2$  is complete  $T_1$  is turned so that side A is in communication with the manometer;  $T_2$  is turned so that on side B the manometer is in communication only with the atmosphere.

The system is again brought to atmospheric pressure and the level of the fluid in the two arms is then at *d*. By a previous calibration the level *c* has been ascertained, which represents the position of the fluid in the two arms when the standard volume of soda is introduced into side A, and the whole brought to atmospheric pressure.

The difference between *c* and *d* is therefore a measure (subject to a small correction) of the volume of carbon dioxide that has diffused across the membrane.

If a correction is required for the diffusion, during  $\text{CO}_2$  estimation, of oxygen from B into A then, after the measurement is complete on the side of A,  $T_1$  is turned so that the manometer on that side is in communication with the atmosphere, while  $T_2$  is also turned so that on that side the manometer is in communication with B. Before the taps are turned the fluid in both limbs of the manometer is at *d*; when the system has been brought to atmospheric pressure the level in the two limbs is at *e*. The difference between *d* and *e* is a measure of the oxygen which has diffused from B into A.

After the membrane had been mounted in the apparatus, but before the experimental gases had been introduced into the opposite sides, the system was tested for leaks by establishing a negative pressure on one side of the membrane when it had air on both sides. The membrane when it is not damaged will sustain indefinitely a pressure of 6 cm. of water. This observation confirms the earlier finding of Wiesner and Molisch that gases cannot be readily forced through seed-coat membranes.

The experiments were all conducted in a chamber in which the temperature was maintained at  $25^\circ \text{C}$ . with a fluctuation of  $0.25^\circ \text{C}$ . on either side. The fluctuations were never sufficiently great to cause any appreciable pressure change in the apparatus.

#### EXPERIMENTAL DATA

The purpose of these experiments was to measure the permeability of the membranes of the seed-coat to each of the three atmospheric gases, and to

analyse the internal conditions that determine the rate at which each of these traverses the membrane.

The second phase of the investigation involves an examination, in terms of diffusion rates, of the effects of various treatments on the membrane itself. The results reported here cover the effects of the following treatments: (a) varying the water content of the membrane, (b) soaking the membrane, (c) exposing to a temperature of 40° C., and (d) exposing to an atmosphere of chloroform.

All the measurements presented here were made with pure gases; the concentration gradient is therefore the steepest possible and always the same. The diffusion rates are given in terms of c.c. per sq. cm. of membrane per hour.

The data of Table I provide a comparison of the rate of diffusion of oxygen, carbon dioxide, and nitrogen both through the inner and outer membranes of the seed. These membranes were separated from seeds that had been soaked for eighteen hours and they were dried for fifteen minutes before use. Each determination in the table represents the results of an individual seed-coat.

TABLE I

*Permeability to Various Gases of Inner and Outer Membranes of the Seed Coat of Cucurbita Pepo; Diffusion as c.c./cm.<sup>2</sup>/hr.*

Outer membranes.			Inner membranes.		
(1)	(2)	(3)	(1)	(2)	(3)
CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>	N <sub>2</sub>
3.2	0.0	0.0	14.3	4.0	3.6
2.5	0.7	0.0	10.5	4.6	3.3
3.2	0.0	0.5	13.5	3.9	2.9
3.0	0.8	0.7	21.3	4.3	3.8
3.4	0.6	0.0	14.9	4.4	2.5
2.8	0.0	0.7	18.4	4.5	3.3
Means	3.0	0.35	15.5	4.3	3.2

In the case of each gas the differences between the means for the outer membrane and inner membrane are statistically significant. It therefore seems that the inner membrane is about five times as permeable to CO<sub>2</sub> and about ten times as permeable to oxygen as the outer membrane. The differences between the carbon dioxide and the oxygen rate and the carbon dioxide and nitrogen rates are significant for both the inner and outer membranes; whereas the differences between the means of oxygen and nitrogen diffusion rates are not. This indicates that carbon dioxide diffuses through the membranes at a considerable greater rate than does oxygen or nitrogen, whereas these two diffuse through the membranes at about the same rate.

The figures of Table I were obtained with membranes which had been exposed for fifteen minutes to the air of the chamber in which the apparatus was used. This treatment it is evident from the figures of Table II was that which ensured a maximum diffusion rate. The data of Table II show the comparative permeabilities to CO<sub>2</sub> and O<sub>2</sub> of inner membranes which had

been (1) used immediately after separation from the cotyledons and not exposed for any appreciable time to the atmosphere, (2) used after exposure to the air of the chamber for fifteen minutes, (3) used after exposure to the air of the chamber for twelve hours. Exposure, of course, reduces the water content of the membrane, and these three series therefore represent the effect of a varying water content on the permeability to gases.

TABLE II

*Effect on the Permeability to CO<sub>2</sub> and N<sub>2</sub> of the Water Content of the Inner Seed-coat Membrane. Diffusion as c.c./cm.<sup>2</sup>/hr.*

Dried 12 hr.		Dried 15 min.		Wet.	
CO <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>
4.0	1.9	14.1	3.9	6.0	0.7
0.9	0.0	15.6	4.4	6.7	1.0
0.5	0.0	14.9	3.7	12.2	0.5
3.1	0.9	18.8	4.2	8.3	0.3
0.9	0.0	17.2	4.2	6.8	1.0
Mean	1.9	16.1	4.1	8.0	1.7

The differences between any two means for carbon dioxide are all statistically significant. The difference between the means for oxygen for membranes exposed for twelve hours and those not exposed are not significant, but both differ significantly from the result for fifteen minutes' exposure. The results show that both gases diffuse most rapidly through the membrane that has been exposed to drying for fifteen minutes, and least rapidly through the membrane that has been dried for twelve hours. The curious fact is also apparent that the saturated membrane occupies an intermediate position, being less permeable than the one that has been dried during a period of fifteen minutes, but more permeable than the one dried for twelve hours.

Table III shows the effect on the permeability of the inner membrane to carbon dioxide and nitrogen of soaking the whole seed for one hour, four hours, and eighteen hours. The membranes with which these measurements were made had been exposed to the air for fifteen minutes before they were mounted in the apparatus.

TABLE III

*Effect of Varying Periods of Soaking the Seed before Removal of the Inner Seed-coat Membrane on the Permeability to CO<sub>2</sub> and N<sub>2</sub>. Diffusion as c.c./cm.<sup>2</sup>/hr.*

Soaked 1 hr.		Soaked 4 hrs.		Soaked 18 hrs.	
CO <sub>2</sub>	N <sub>2</sub>	CO <sub>2</sub>	N <sub>2</sub>	CO <sub>2</sub>	N <sub>2</sub>
9.3	2.4	11.3	2.3	14.1	3.5
10.3	3.1	12.2	2.7	15.6	3.0
6.2	2.2	10.2	1.0	15.0	2.8
8.1	1.6	12.8	3.0	18.9	3.7
9.6	1.5	11.8	2.4	17.2	2.1
Means	8.7	11.7	2.3	16.2	2.9

The means between the nitrogen series do not differ significantly but the corresponding differences between any two carbon dioxide means are all significant. From this it is evident that lengthening the period of soaking increases the permeability of the inner membranes to carbon dioxide.

The data collected in Table III demonstrate the effect of soaking a membrane while it is still attached to the seed. The possible influence of the cotyledons on this effect was tested by comparing the rates of carbon dioxide and nitrogen diffusion through membranes which had been soaked after they had been detached from the seed with others which had been soaked for the same period while still attached to the cotyledons. The results are presented in Table IV. For the series representing the permeability to carbon dioxide and nitrogen of detached membranes, the cotyledons were soaked for an hour before removal of the membranes, and the latter were then soaked for a further three hours. The membranes for the second series were detached from cotyledons which had been in water for four hours. All the membranes were dried for fifteen minutes before use.

TABLE IV

*Effect of Soaking in Different Ways on the Permeability of the Inner Seed-coat Membrane. Diffusion as c.c./cm.<sup>2</sup>/hr.*

Membranes attached to cotyledons during soaking.		Membranes soaked after removal from cotyledons.	
CO <sub>2</sub>	N <sub>2</sub>	CO <sub>2</sub>	N <sub>2</sub>
12.0	2.4	15.6	7.6
11.5	2.0	14.0	3.6
9.6	1.5	14.0	2.5
12.4	2.8	16.8	3.1
10.5	2.1	13.2	4.1
10.9	1.8	15.1	3.0
Means	11.2	14.8	4.0

The differences between the two sets of means are statistically significant for both carbon dioxide and for nitrogen. These figures indicate that the effect is greater when the membranes are soaked after being detached from the cotyledons.

Previous anatomical observations had suggested the possibility that many of the characteristic reactions of these inner membranes might be due to one of the layers of cells being living. The effect of destroying the living cells was therefore tested. The data of Table V demonstrate the effect of treatment with chloroform vapour for two minutes and those of Table VI that of pre-treatment with a temperature of 40° C. for five minutes. The membranes for this experiment were detached from seeds which had been soaked for eighteen hours, and then dried for fifteen minutes before pre-treatment.

TABLE V

*Effect of Treatment with Chloroform Vapour on the Permeability of the Inner Seed-coat Membrane. Diffusion as c.c./cm.<sup>2</sup>/hr.*

Control.		Treated with chloroform.	
CO <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>
11.4	3.0	24.0	4.0
13.1	6.8	29.8	4.5
12.1	3.0	25.1	3.7
13.2	4.0	29.6	5.1
16.7	4.0	28.8	4.5
12.1	3.7	26.2	3.8
15.7	4.1	27.3	4.3

TABLE VI

*Effect of Treatment at 40° C. on the Permeability to Gases of the Inner Seed-coat Membrane. Diffusion as c.c./cm.<sup>2</sup>/hr.*

Control.		Exposure to 40° C.	
CO <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>
13.1	3.8	20.8	5.1
11.4	3.6	18.4	4.0
15.4	3.9	19.7	4.4
16.5	4.7	19.8	5.4
14.2	3.6	18.5	4.9
15.2	3.7	21.8	4.9
14.3	3.9	19.8	4.8

The differences between the means for carbon dioxide are significant for both chloroform and heat treatment. Those between the means for oxygen are not. This indicates that although pre-treatment does not significantly affect the permeability of the inner membranes to oxygen it is significant in regard to carbon dioxide.

#### DISCUSSION OF RESULTS

Most of the observations described in this paper relate to the inner membrane. This restriction was suggested by experiments which showed that this membrane has a very profound effect on both the respiration and on the germination rate of the seed. The restriction is also justified by the probable predominant influence of the inner membrane on the gaseous exchange of the seed.

The two membranes of the seed are separated by an air-gap which is traversed at intervals by groups of very thin-walled cells, identified by von Höhnelt (1876) as the remains of the inner integument. This space is occupied by air not only in the dry but also in the imbibed seed. Even after seeds have been completely immersed in water for six hours air can still be withdrawn from them by subjecting them while still immersed to a reduced pressure;

small bubbles then stream out of the micropyle. That the gas does not come from the cotyledons or the embryo is shown by repeating the experiment after removal of the outer membrane; no gas then appears. It would therefore seem that until the enveloping membranes of the seed are broken during the later stages of germination the embryo and cotyledons are surrounded by an air-jacket.

The presence of this air-jacket is of some considerable importance in relation to the gaseous exchange between the seed and its environment, for it necessitates a gaseous exchange in two stages. There must first be an exchange between the embryo and cotyledons and the air-gap between the two membranes, and secondly an exchange between the air-gap and the outer environment. The rate at which the whole process is maintained will depend on the resistances to diffusion in each of the two parts of the system. Now it is evident from the data of Table I that the resistance of the outer membrane to gaseous diffusion is very much greater than that of the inner, so that if the gaseous exchange between the atmosphere and the air-gap depended solely on diffusion across the outer membrane, it would be the resistance to diffusion within this membrane which determined the rate of the whole process. The inner and outer membranes, however, differ, for whereas the inner membrane envelops the seed completely, the continuity of the outer is interrupted by the micropyle. Thus, although the resistance of the outer membrane to gaseous diffusion is greater than that of the inner it cannot control the gaseous exchange so long as the micropyle remains open. That the micropyle is not obstructed is shown by the experiment described above, in which seeds immersed in water were subjected to a reduced pressure. Moreover, the characteristics of the respiration process of the seed preclude the further possibility of blocking by water. As Stiles and Leach (1932) have shown, the value of the respiratory quotient of these seeds is always less than unity, which means that more oxygen is being absorbed than carbon dioxide evolved. This must occasion a reduced pressure in the air-gap which would break any film of water which might form across the micropyle under all except waterlogged conditions. A reduced pressure in the air-gap also would result in a mass flow of gas through the micropyle. Thus the exchange between the air-gap and the atmosphere probably takes place by diffusion along a free gaseous path through the micropyle supplemented by a mass flow of gas along this same channel. The rates of entry through the micropyle will of course depend on the rate of diffusion across the inner membrane, since the resistance of the inner membrane is greater than that established along a free gaseous path in the ratio of about 8,500:1. The inner membrane must therefore be the controlling factor in the exchange of gases between the seed and its environment.

The morphological and anatomical characteristics of the enveloping membranes of the seed of *Cucurbita* were studied in great detail by von Höhnelt (1876). According to him the part of the seed-coat that comprises what has

been called in this paper the 'inner membrane' consists of five tissue layers: (a) an outer layer of dead thin-walled cells containing chlorophyll, derived from the inner integument of the ovule; (b) a one-celled layer, having thick walls, the outer surface of which is cutinized, representing the epithelium of the 'nucellus'; (c) a many-layered tissue consisting of dead, thin-walled, highly compressed cells that represent the remains of the nucellus; (d) a one-celled layer with thick walls enclosing a dense mass of oil drops and protein granules—the outermost layer of the endosperm; (e) a tissue consisting of dead, thin-walled, highly compressed cells which are the remains of the digested endosperm.

The structure of this membrane was also examined by Marei (1919), who confirmed substantially the description of it given by the earlier worker. He was unable, however, to identify the layer (b) although he figured a cuticle in relatively the same position as that assigned to it by von Höhnell. Some observations made by the author on the anatomical structure of the inner membrane confirm von Höhnell's description in all particulars. When the membrane is soaked in water the walls of layer (b) may swell to such an extent as to obliterate the lumen; this may be the reason for Marei's failure to identify this layer.

The inner membrane is thus a complex structure, so the passage of gases across it cannot be compared with that of an homogeneous non-living membrane of which the permeability to gases can be accounted for in simple physico-chemical terms. Much of the resistance of the inner membrane must indeed be due to the physico-chemical characteristics of its component dead layers; but much of it, as the data of Tables V and VI suggest, must also be due to the living nature of one or more of these tissue components. These two causes of resistance to diffusion are largely independent of each other, and as such they will now be considered.

The structural differences in the several tissue-layers make this membrane comparable with one formed by superimposing successive layers each chemically and physically distinct. Thus, although the differences between the rates at which various gases traverse a simple membrane may be related directly to their solubilities or to their diffusive properties depending on their densities, no such simple differential behaviour of gases can be expected here. Both factors are doubtless operative, but that the rate of the passage of the gas is not determined by any simple diffusion process is indicated by the want of relationship between the rates at which the gases traverse the membrane and their densities. Moreover, the observation that gases cannot be forced through under pressure indicates that the membrane is traversed by no pores larger than molecular dimensions; there are therefore no channels along which free gaseous diffusion can be maintained. Nevertheless, there are in the body of the membrane gas-filled spaces, such as those of the inter-cellular spaces, and of the lumina of dead cells; across these gases must diffuse in their passage through the membrane, and in these the conditions for free

gaseous diffusion must obtain. The effect of this phase in the process is discussed in a later paragraph.

Several facts suggest a dominating influence on the rate at which the gases traverse the membrane of their solubility in the component substances. These facts are: (1) the absence of an overmastering effect of free diffusion, (2) the effect of the water content on the permeability of the membrane to gases, and (3) the rate at which carbon dioxide traverses the membrane as compared with that of the other two gases studied. The figures of Table II suggest that water must form an important vehicle for the transport of gases across the membrane. In the tissues of the dry membrane the gases are evidently insoluble; as soon, however, as these tissues absorb water they become permeable by virtue of the solubility of gases in water. This suggestion is also supported by the data on the rates at which carbon dioxide diffuses across the inner membrane. The much higher value given by this gas as compared with those given by nitrogen and oxygen reflects its relatively greater solubility in water. Water, however, cannot be the only solvent concerned in the transmission of gas; it can only be operative in those components of the membrane by which it is absorbed. There is at least one continuous layer, that of the cuticle, which is hydrophobic. The extent to which the solubility factor controls the rates at which a gas traverses the membrane will therefore depend on solubility both in water and in the fatty constituents of the cuticle.

The experimental evidence for the influence of free gaseous diffusion is provided by the data on the permeability of saturated and of partially dried membranes presented in Table II. The fully saturated membrane is one in which not only the walls are saturated but in which also the lumina and intercellular spaces are occupied by free water. In the process of drying, this free water, which is held between the members of the solid framework, must of necessity be the first to be withdrawn by evaporation. The water in the colloidal walls is held by imbibitional forces which will prevent any loss until all free water has been withdrawn. The partially dried membrane will therefore differ from the saturated membrane in the replacement by air of the water in the intercellular spaces and in the cell lumina. The wet but not saturated membrane has thus tracts along which the gas can move more rapidly than through water. Hence the greater permeability of the membrane which has been dried for fifteen minutes as compared with that from which only surface moisture has been removed.

The absorption of water, as has been shown, immediately increases the permeability of the membrane by providing a medium in which the gases can dissolve. It is evident, however, from the data of Table III that this absorption of water has also a secondary effect which is only apparent after a lapse of some time. The increasingly greater permeability of the inner membrane as the time of soaking is increased might be due to one or more of the three following effects: (1) During soaking there may be changes of a chemical nature leading to the formation of substances with a higher imbibitional



pressure, which, after the drying for fifteen minutes, would result in a greater retention of water and thus a greater permeability to gases. This possibility is, however, excluded by the fact that the water content of membranes dried for fifteen minutes is always between 60 and 65 per cent. of the wet weight whatever the soaking treatment. (2) It was thought that the effect of soaking might be due to the stretching of the membrane consequent on the swelling of the seed. That this is not so is shown by the data of Table IV which demonstrates that soaking after separation from the cotyledon induces an even more rapid increase in permeability. (3) The third possibility that soaking increases the permeability of the membrane to gases by removing in solution some comparatively impermeable constituent substances seems from the data of Table IV the most probable. When the membrane is attached to the cotyledons the dissolved substances can only diffuse in one direction; when, on the other hand, it is separated from the rest of the seed, diffusion can proceed from both surfaces, and thus move rapidly.

The data of Tables V and VI suggest that in an analysis of the conditions which control the rate of diffusion of gases across the membrane a purely biological factor must also be considered. Exposure to chloroform vapour or to a temperature of 40° C. is usually lethal to living cells, and the result of these treatments indicates that the increased permeability is due to the death of a component of the membrane. Evidence of the presence of living layers in seed-coat membranes has been presented by a number of earlier workers. Crocker (1906), on the basis of microscopical examination, identified as living the three innermost layers of the seed-coat of *Xanthium*. Becquerel (1907) and later Shull (1913) both observed a vigorous respiratory activity in the seed-coats of a number of species. The author has also found that the inner membrane of the seed-coat of *Cucurbita* respire. It would seem that the part of the inner membrane which is responsible for this is the layer (*d*) which was identified by von Höhnelt as the outermost, and the only undigested, layer of the endosperm. This observation of von Höhnelt's makes this layer morphologically homologous with the aleurone layer of cereal grains. The two are in fact remarkably similar in appearance, the layer in *Cucurbita* having the same dense granular content of hyaline protein granules. Further evidence of the living condition of its cell contents appears in the fact that after soaking in water for some forty-eight hours the cells of the layer can then be plasmolysed.

#### SUMMARY

1. An apparatus is described by which the absolute rates of diffusion of gases across the seed-coat can be measured.
2. The seed-coat of *Cucurbita* consists of two membranes, of which the outer is much less permeable to gases than is the inner. It is the inner, however, that controls the gaseous exchange since the outer is perforated by the micropyle.

3. The mean values for diffusion in c.c.<sup>3</sup> cm.<sup>2</sup> hr. are as follows: outer membrane, CO<sub>2</sub> 3.0, O<sub>2</sub> 0.35, N<sub>2</sub> 0.31; inner membrane, CO<sub>2</sub> 15.5, O<sub>2</sub> 4.3, N<sub>2</sub> 3.2.

4. The absorption of water increases the permeability to gases of the inner membrane by (1) providing a medium in which the gases can dissolve, (2) removing in solution some impermeable constituent.

5. The saturated membrane is less permeable than one which has been partially dried, since in the first the lumina and intercellular spaces are occupied by water, whereas in the second free paths for diffusion exist.

6. There is in the inner membrane a living layer in which the resistance to diffusion is high. When this layer is killed the rate of diffusion across the whole membrane increases considerably.

7. The structure of the seed-coat is described and its bearing on the problem under consideration is discussed.

The author wishes to acknowledge his indebtedness to Prof. F. G. Gregory; the work was done under his direction and owes much to his constant interest and advice.

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# The Factors Determining the Resistance to the Movement of Water in the Leaf

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With eight Figures in the Text

THE total resistance to the movement of fluid water in a leaf comprises the resistance in the main veins, that in the fine veins and the vessel endings, and that through the protoplast into the mesophyll cells.

It has been shown by Huber (1928) that the resistance to movement of water through the vascular system of the stem is not very great, while the resistance to the passage of water through the protoplast is of a very high order (Baptiste, 1935; De Haan, 1933; Huber and Höfler, 1930; McCutcheon and Lucke, 1928; Palva, 1939).

It was thought desirable, therefore, to determine the relationship between the resistances of the various parts of the water channels of the leaf.

## EXPERIMENTAL

A leaf was detached from the plant (*Pelargonium zonale* var. *Paul Crampel*), fitted into a potometer (Gregory, 1938), and allowed to stand overnight in a temperature-controlled greenhouse where the experiment was subsequently carried out. During the experiment the leaf was illuminated by a water-screened lamp arranged to give 100 ft.-candles at the leaf surface.

The absorption of water was followed, and the effect on it of cutting the midrib and parts of the lamina was observed. The results of two experiments (expts 1, 2) are shown graphically in Figs. 1 and 2, in which absorption in mg./min. is plotted against time, the arrows indicating the times at which the incisions were made, while their positions are indicated in Figs. 3 and 4.

It was expected that the interference with the vascular system of the lamina would result in a fall in the amount of water absorbed, due to the isolation of the distal sector of the leaf from its direct supply of water. Reference to Figs. 1 and 2 shows that this is not so, indicating that the supply of water to the isolated sector must be carried with equal adequacy through the network of xylem connecting this sector to the nearest intact main vein. Wylie (1938) has previously recorded a similar conclusion.

If the alternative path has a very high resistance as compared with the main veins, then, in order to maintain an unchanged flow of water, the suction

tension of the isolated section of the leaf must be raised to overcome this increased resistance. This could be accomplished if, during the period after severing the midrib, there was a fall in the water content of the isolated portion of leaf.

The previous results indicated that there was no decrease in amount of water absorbed after the experimental treatment; the following experiments

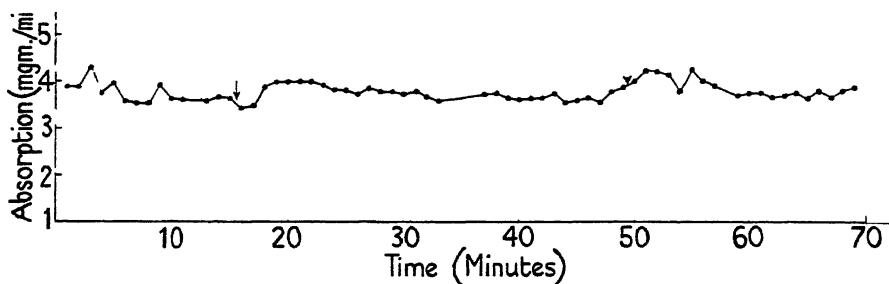


FIG. 1. Result of exp. 1 showing absorption plotted against time. The first arrow indicates the time at which the midrib was cut (A fig. 3) the second refers to the lamina incisions (B fig. 3).

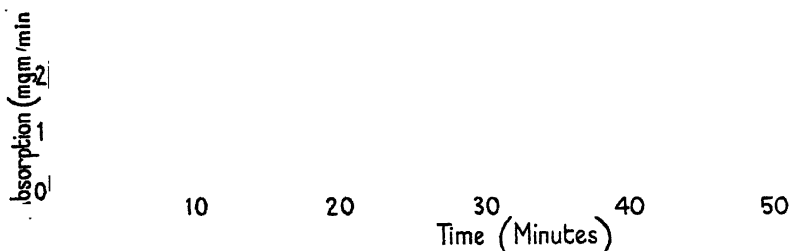


FIG. 2. Result of expt. 2 showing absorption plotted against time. The arrows indicate the successive times at which the midrib and lamina were cut (fig. 4 midrib, A, B, C.)

were therefore carried out to determine if there was any change in the rate of transpirational loss.

The leaf, fitted into a potometer, was attached to one arm of a balance from which the pan had been removed. The balance was damped, so that as the leaf transpired, the pointer moved slowly across the scale to the zero position. At this point a stop-watch was started, and 15 secs. later the potometer reading was taken. The balance rider was then moved to a position corresponding to a loss in weight of 10 mg., and the time for the pointer to reach the zero mark again was observed and 15 secs. later the potometer reading again taken. Thus data of the transpiration and absorption over the same period, staggered by 15 secs. only, were obtained, and by using two stop-watches continuously recorded.

The results of two experiments (expts. 3, 4) are shown in Figs. 5 and 6, in which both absorption and transpiration (mg./min.) are plotted against time. In these experiments the midrib alone was severed, the incision being made

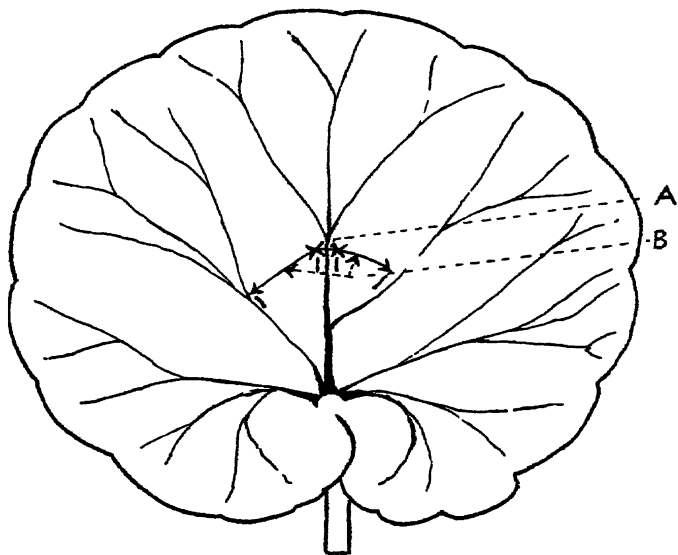


FIG. 3. The positions of the incisions made in expt. 1 (Fig. 1) are shown. The midrib was cut at A and two cross incisions made as shown at B. The extent of the cuts is emphasised by the short lines below the arrowheads.

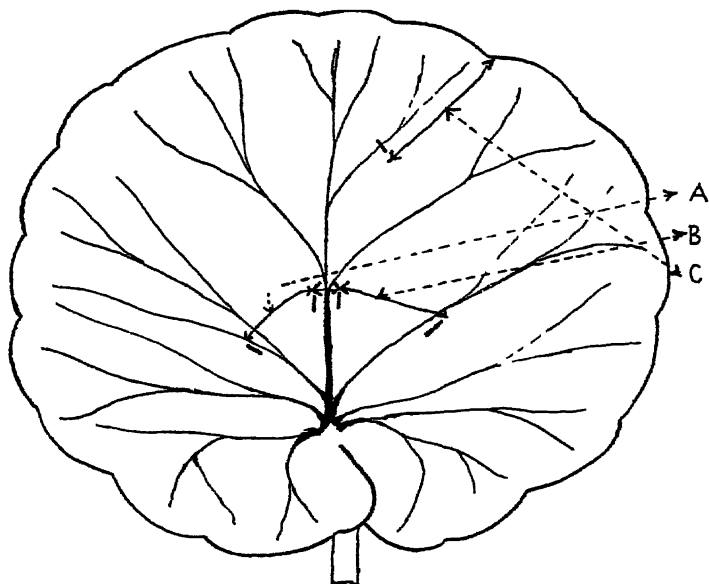


FIG. 4. The positions of the incisions made in expt. 2 (Fig. 2) are shown. In this case the midrib was cut as in Fig. 3, expt. 1, and additional incisions made in the positions indicated by the dotted lines A, B, and C.

approximately half-way between the distal end of the lamina and the junction with the petiole, and at the time indicated by an arrow in the graph.

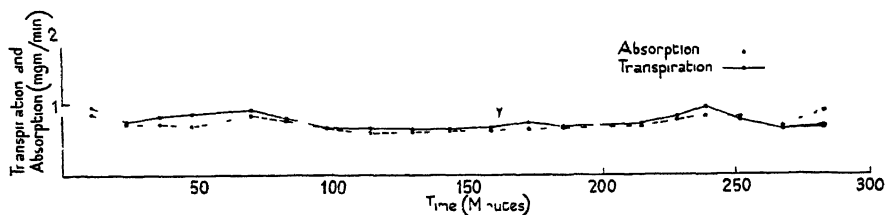


FIG. 5.

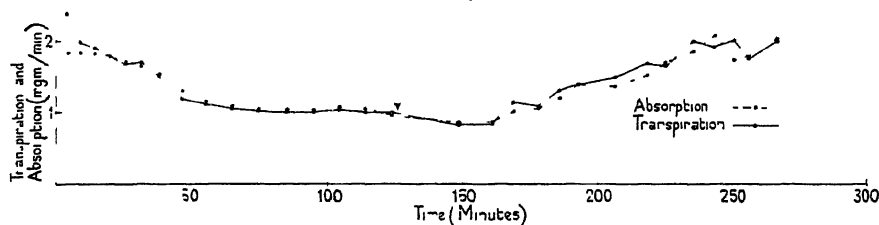


FIG. 6.

FIGS. 5 and 6. Results of expts. 3 and 4 respectively showing transpiration and absorption plotted against time. The arrow indicates the time at which the midrib was severed.

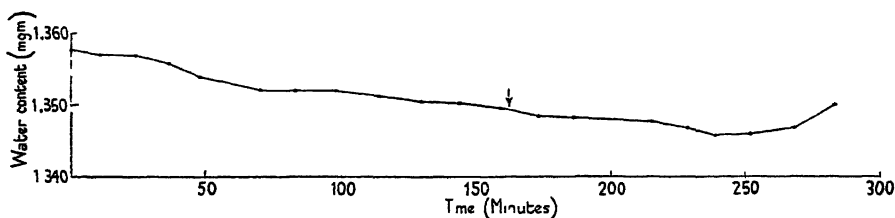


FIG. 7.

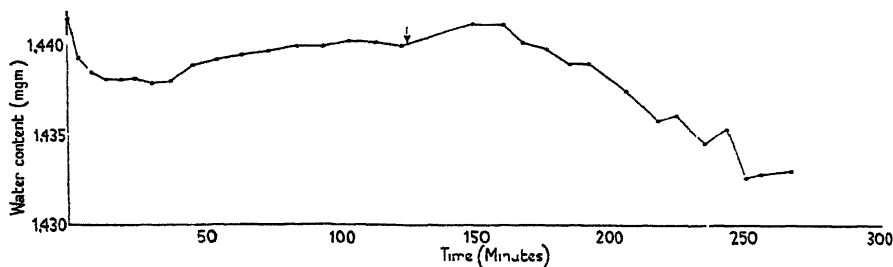


FIG. 8.

FIGS. 7 and 8. The water content of the leaves used in expts. 5 and 6 are shown.

FIGS. 7 and 8 represent the water content of the leaves corresponding to FIGS. 5 and 6 respectively. It will be seen from FIGS. 5 and 6 that there is no sudden change in transpiration loss following the severance of the midrib.

Fig. 7 shows a gradual fall in water content, implying a corresponding rise in the suction tension of the leaf. Ultimately, however, the water content is slightly higher than at the time when the incision was made. Fig. 8 shows a slight increase in the water content after cutting the midrib, followed by sustained fall, the decrease in water content at the end of the experiment being approximately 0.5 per cent. of that at the time of making the incision.

If an increase in suction tension followed the experimental treatment of the leaf, this would be indicated by a fall in water content immediately after cutting the vein. It is doubtful, therefore, if the gradual changes in water content shown in Figs. 7 and 8 are due to the interference with the vascular system of the leaf. They may most probably be referred to other physiological processes in operation during the experimental period.

### CONCLUSION

It may be concluded, therefore, that there is no increase in the suction tension of the leaf, or that it is experimentally indeterminate, indicating that the minor veins offer no considerable resistance to the passage of water, and that the main resistance to the movement of water in the leaf must therefore be encountered in the passage through the protoplast of the mesophyll cells; in comparison with this resistance that of the vascular system is negligible.

The experiments here described were suggested by Professor F. G. Gregory to whom the author wishes to express his thanks.

### SUMMARY

The effect of severing the midrib and parts of the lamina on the absorption and transpiration of leaves is described, and the conclusion is drawn that the resistance to the movement of water through the net-veins of the leaf is very small compared with the resistance to the passage of water out of the veins into the mesophyll cells.

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# Physiological Studies in Plant Nutrition

## X. Water Content of Barley Leaves as Determined by the Interaction of Potassium with certain other Nutrient Elements

### PART II. *The Relationship between Water Content and Composition of the Leaves*

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### INTRODUCTION

IN the first part of this paper (Richards, 1940) an experiment was described in which barley was grown with twenty-two nutrient combinations, the nutrient variables being potassium (3 levels:  $K_1$ ,  $K_3$ , and  $K_5$ ), phosphorus (2 levels: H and L), and the sodium-calcium ratio (5 combinations: A, B, C, D, and E). For convenience of reference the nomenclature scheme is repeated under Table I. Samples were taken at three periods during the main part of the vegetative cycle. The water-content data for green leaves and stems presented there were submitted to an analysis of variance and the significant first order effects and interactions between treatments and sampling times were defined. There will now be presented the results of an attempt to assess statistically the individual effects of the internal contents of the variable elements in the experiment on the water content of the green leaf.

### RELATIONSHIP OF WATER CONTENT TO CONTENTS OF INDIVIDUAL ELEMENTS

1. *Relationships between treatments.* Table I gives the green-leaf contents of these elements as percentage  $K_2O$ ,  $Na_2O$ ,  $CaO$ , and  $P_2O_5$  of the silica-free dry

[Annals of Botany, N.S. Vol. IV, No. 15, July 1940.]

matter.<sup>1</sup> These data will be discussed only in so far as they throw light on the water-content data. The problem as to how far the observed variations in

TABLE I

*Leaf Contents of Mineral Elements (per cent. Silica-free Dry Weight)*

Treatment.	Sample 1 (32-4 days)				Sample 2 (39-41 days)				Sample 3 (53-5 days).			
	K <sub>2</sub> O	Na <sub>2</sub> O	CaO	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	Na <sub>2</sub> O	CaO	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	Na <sub>2</sub> O	CaO	P <sub>2</sub> O <sub>5</sub>
HAK <sub>1</sub>	4.70	5.34	0.82	1.80	3.15	5.12	0.91	1.20	1.44	2.19	0.58	0.52
HAK <sub>3</sub>	1.42	4.30	0.97	1.64	0.55	4.82	0.90	1.32	0.33	4.86	0.49	1.06
HAK <sub>5</sub>	0.63	9.47	1.01	2.04	0.64	8.87	0.93	1.54	0.66	6.84	0.67	1.41
HBK <sub>1</sub>	4.64	3.29	1.46	1.31	2.88	2.75	1.41	0.83	1.22	1.85	0.74	0.44
HBK <sub>3</sub>	1.80	3.08	1.79	1.27	0.56	3.53	1.45	0.71	0.31	4.32	0.87	0.63
HBK <sub>5</sub>	0.60	7.00	1.71	1.52	0.64	7.48	1.40	1.30	0.65	6.27	0.66	1.09
HCK <sub>1</sub>	3.62	0.47	2.32	1.10	2.42	0.38	2.27	0.80	0.94	0.27	1.12	0.39
HCK <sub>3</sub>	1.29	0.64	2.32	1.03	0.52	0.56	2.76	0.71	0.44	0.58	2.92	0.54
HCK <sub>5</sub>	0.27	0.78	3.52	1.35	0.31	1.48	2.20	1.26	0.39	1.93	1.64	1.12
LAK <sub>1</sub>	6.45	3.85	0.90	0.86	4.91	3.55	0.46	0.48	4.36	2.62	0.56	0.27
LAK <sub>3</sub>	1.76	5.52	0.51	0.72	0.68	4.09	0.57	0.50	0.44	3.09	0.63	0.29
LAK <sub>5</sub>	0.93	7.08	0.76	1.31	0.77	8.02	0.56	0.91	0.67	7.15	0.84	0.75
LBK <sub>1</sub>	5.44	2.26	1.46	0.64	4.56	1.85	1.16	0.46	2.68	0.98	1.13	0.30
LBK <sub>3</sub>	1.72	4.15	1.33	0.75	0.71	3.33	1.04	0.47	0.42	2.47	1.03	0.32
LBK <sub>5</sub>	0.90	5.72	1.79	0.78	0.80	5.32	1.18	0.48	0.59	4.13	1.19	0.47
LCK <sub>1</sub>	4.01	0.75	2.16	0.70	3.69	0.40	1.65	0.44	2.42	0.28	1.54	0.23
LCK <sub>3</sub>	1.58	1.16	2.95	0.69	0.76	0.88	2.15	0.44	0.61	0.33	1.57	0.28
LCK <sub>5</sub>	0.68	1.34	2.93	0.68	0.43	1.35	3.19	0.52	0.48	1.28	3.01	0.47
HDK <sub>5</sub>	0.32	5.66	2.70	1.44	0.32	4.47	1.88	1.18	0.38	4.12	1.49	1.14
LDK <sub>5</sub>	0.74	4.09	2.42	0.72	0.74	4.20	1.83	0.54	0.48	3.11	1.54	0.48
HEK <sub>5</sub>	0.49	7.33	1.41	1.49	0.49	8.01	0.93	1.31	0.66	6.70	0.86	1.22
LEK <sub>5</sub>	0.82	6.09	1.81	0.75	0.82	5.61	1.10	0.57	0.59	4.90	1.03	0.49

*Nomenclature*

	High sodium. Low calcium.	Medium sodium. Medium calcium.	No sodium. High calcium.	
High potassium	HAK <sub>1</sub> LAK <sub>1</sub>	HBK <sub>1</sub> LBK <sub>1</sub>	HCK <sub>1</sub> LCK <sub>1</sub>	High phosphorus Low phosphorus
Medium potassium	HAK <sub>3</sub> LAK <sub>3</sub>	HBK <sub>3</sub> LBK <sub>3</sub>	HCK <sub>3</sub> LCK <sub>3</sub>	High phosphorus Low phosphorus
Low potassium	HAK <sub>5</sub> LAK <sub>5</sub>	HBK <sub>5</sub> LBK <sub>5</sub>	HCK <sub>5</sub> LCK <sub>5</sub>	High phosphorus Low phosphorus
	Low sodium. High calcium.	High sodium. Medium calcium.		
Low potassium	HDK <sub>5</sub> LDK <sub>5</sub>	HEK <sub>5</sub> LEK <sub>5</sub>		High phosphorus Low phosphorus

<sup>1</sup> Standard micro-analytical methods were used. After ashing, potassium was precipitated with sodium cobalti-nitrite and estimated by a slight modification of the method of Beneditti-Pichler (1924). Sodium was estimated as sodium magnesium uranyl-triacetate, adopting with modifications the process of Kahne and Dumont (1932). Calcium was precipitated in the presence of acetic acid and weighed as hydrated oxalate. Finally, phosphorus was estimated as ammonium phospho-molybdate, using the method of Pregl (Roth, 1937).

succulence may be accounted for by the differences in content of these elements is best examined by means of the analysis of co-variance. The twenty-two treatments and three samples provide sixty-six sets of observations for the determination of correlation or regression coefficients, and the total relationships may be analysed adequately for the immediate purpose into relationships between treatments, those between sampling times, and those within the interaction of treatments with time. The resulting correlation coefficients, total, partial, and multiple, for the experiment considered as a whole, and for the sample totals, are given in the first two columns of Table II.

TABLE II

*Correlation Coefficients between the Variables Water Content (W), Potassium Content (K), Sodium Content (Na), Calcium Content (Ca) and Phosphorus Content (P)*

Total and partial coefficients in italics exceed the 5 per cent. point in significance.  
Total and partial coefficients in heavy type exceed the 1 per cent. point in significance.  
Multiple coefficients in italics indicate that each independent variate makes a significant contribution.

	Whole experiment.	Sample totals.	Sample 1.	Sample 2.	Sample 3.
Total coefficients:					
WK	-0.216	-0.418	-0.271	-0.365	-0.386
WNa	+0.871	+0.913	+0.868	+0.910	+0.925
WCa	-0.286	-0.415	-0.479	-0.472	-0.295
WP	+0.725	+0.743	+0.635	+0.670	+0.865
Partial coefficients:					
WK·Na	+0.003	-0.260	+0.018	-0.211	-0.304
WK·Ca	-0.279	-0.630	-0.528	-0.572	-0.482
WK·P	-0.222	-0.241	-0.211	-0.220	-0.062
WNa·K	+0.864	+0.901	+0.857	+0.900	+0.920
WNa·Ca	+0.880	+0.922	+0.828	+0.900	+0.942
WNa·P	+0.807	+0.871	+0.818	+0.866	+0.897
WCa·K	-0.334	-0.628	-0.633	-0.630	-0.420
WCa·Na	+0.379	+0.507	+0.131	+0.374	+0.531
WCa·P	-0.390	-0.392	-0.412	-0.509	-0.293
WP·K	+0.726	+0.699	+0.620	+0.629	+0.840
WP·Na	+0.561	+0.594	+0.446	+0.447	+0.812
WP·Ca	+0.749	+0.736	+0.598	+0.689	+0.865
WK·NaCa	+0.158	+0.178	+0.163	+0.037	-0.088
WNa·CaP	+0.779	+0.879	+0.779	+0.827	+0.920
WCa·NaP	+0.215	+0.449	+0.116	+0.257	+0.530
WP·NaCa	+0.487	+0.552	+0.442	+0.362	+0.811
WK·NaCaP	+0.050	+0.222	+0.159	+0.012	+0.130
Multiple coefficients:					
W·NaCa	0.890	0.936	0.871	0.923	0.947
W·NaP	0.913	0.945	0.896	0.928	0.975
W·NaCaP	0.917	0.956	0.897	0.933	0.982

Total and partial coefficients exceeding the 5 per cent. point in significance are in italics and those exceeding the 1 per cent. point in heavy type; all the multiple coefficients are, of course, significant, but those given in italics are coefficients in which every one of the independent variates concerned is shown by the partials to contribute significantly. In the remaining three columns are given the corresponding coefficients for the individual samples, which may be conveniently considered with the treatment effects derived from the analysis of co-variance, though properly their differences belong to interaction effects.

The entries in all five columns are in general remarkably consistent in assessing the importance of the various elements for the purpose of prediction of water content, and it is evident that most of the variation in succulence between treatments may be accounted for in terms of the internal concentrations of these elements. The high values of the multiple coefficients derived from the entire sixty-six sets of observations considered together, and the similar values for all coefficients derived from the data of the individual samples, or sample totals, show that by far the greatest part of the total observed differences in water content is closely related to these internal factors (potassium, sodium, calcium, and phosphorus contents) as determined by the nutritional treatments; hence on completing the analysis of co-variance any remaining relations which may be established between succulence and the inorganic elements can contribute only a small fraction to the total variance in water content and, so far as this experiment is concerned, constitute only minor effects.

In reviewing the results in Table II, the two most striking facts which emerge are (1) the general lack of correlation with potassium content, and (2) the equally uniform close correlations with sodium content. All total correlations with potassium content are negative and low. On eliminating effects ascribable to sodium (WK·Na) or phosphorus (WK·P) content they remain substantially unchanged, but if calcium content be eliminated (WK·Ca) in every case the coefficient becomes more negative and reaches significance level. The inverse nature of the relationship probably indicates that this correlation is not due to a direct effect of potassium, and, in fact, further analysis reveals it to be wholly spurious. For the further elimination of the effect of sodium content (WK·NaCa) changes the coefficients to low positive values, which are unaltered by the final elimination of effects related to phosphorus content (WK·NaCaP). The introduction into the correlations of the square and higher powers of potassium content also fails to support the theory of the existence of a simple and important connexion between the element potassium and water content.

*There is, then, between treatments, no statistical evidence of any appreciable effect on succulence of potassium content as such*, though the final partials, with very low positive values, may reflect some slight relationship. This result, if surprising, is all the more convincing when it is considered that very wide ranges both of succulence and of potassium content have been explored, hence

the experimental conditions are favourable for discovering any relation, should such exist. Moreover, so soon as hypotheses are abandoned that postulate a regulatory effect of potassium content on water content, the way is clearly open for an explanation of the inconsistencies in the results of different investigators on the effects of potassium deficiency (see Richards and Shih, 1940).

Considering the slight effect attributable to potassium content, the very close relation revealed everywhere in Table II between sodium content and succulence is also remarkable. The coefficients, both total and partial, are always high and positive, and sodium clearly accounts for the greatest part of the variation in water content between treatments. A very wide range of content is again explored, and from Table I it is seen that in the high sodium treatments, under extreme potassium deficiency ( $AK_5$ ), contents of  $Na_2O$  up to about 9 per cent. of the dry weight may be obtained, accounting readily for the great increase in succulence under these conditions. The relationship between sodium and water contents appears to be nearly linear; in fact, only in sample 1 is there appreciable evidence that the departure from linearity may be real; this will be referred to later when the phosphorus correlations are described.

The total and partial correlations between calcium and succulence in some respects resemble those already described between potassium and succulence. Total coefficients are everywhere negative, three of them even exceeding in significance their 5 per cent. points, and on eliminating phosphorus they are substantially unaltered. The elimination of potassium ( $WCa \cdot K$ ) leads to larger negative values, all but that of sample 3 now exceeding 1 per cent. significance. By eliminating sodium ( $WCa \cdot Na$ ), on the contrary, the signs are reversed and the coefficients increase from a low value at sample 1 to significance level at sample 3. The correlations derived from the sample totals and from the whole experiment are also significant. These are again reduced somewhat by the further elimination of phosphorus ( $WCa \cdot NaP$ ), but there appears to be an undoubted relationship in the third sample. It is interesting to note that only after the effect of sodium has been eliminated does a reversal of direction of correlation with calcium and potassium from negative to positive occur, and a positive relation must hold if these elements help to determine water content through osmotic effects; this fact stresses the primary importance of sodium content in the experiment.

Finally, the total coefficients between phosphorus content and succulence are all high, exceeding in significance their 1 per cent. points, nor are they appreciably lowered on eliminating either potassium or calcium. But the elimination of sodium effects ( $WP \cdot Na$ ) leads everywhere to a considerable fall in correlation; similarly the elimination of phosphorus effects from the total sodium relationship ( $WNa$  and  $WNa \cdot P$ ) results in an appreciable lowering of the coefficient. Now phosphorus and sodium contents are themselves highly positively correlated, hence the fact that the partials are lower than the total coefficients does not mean that spurious effects alone have been eliminated,

but rather that it is impossible from the data to assess accurately the relative parts played by the two elements individually. The interdependence of sodium and phosphorus is, however, insufficient to obscure a statistically significant partial correlation between phosphorus content and succulence. But more obscuring interdependence among the variates is revealed when the attempt is made to discriminate between phosphorus and calcium. It was mentioned in the preceding paragraph that if from the partial  $WCa \cdot Na$  phosphorus content is further eliminated ( $WCa \cdot NaP$ ) the result is a lowering of all coefficients. Similarly, the elimination of calcium ( $WP \cdot NaCa$ ) from the partial  $WP \cdot Na$  lowers all coefficients, some but slightly, others (in particular, sample 2 and the whole experiment) appreciably. It may be stated, therefore, that in general phosphorus and calcium effects accompany the sodium effect. While it is impossible always to discriminate sharply between the three effects, very clear evidence is presented by the data for sample 3. Since the three partials  $WNa \cdot CaP$ ,  $WP \cdot NaCa$ , and  $WCa \cdot NaP$  entered in all five columns of Table II always appear in that order as a series of decreasing magnitude, there is consistent evidence that throughout the experiment the importance of the elements in relation to water content fall into the order sodium > phosphorus > calcium. Even in sample 3, where the evidence is clearest and a calcium effect is demonstrated, it is, however, of small magnitude. Thus in the multiple correlation including all three independent variables, the contribution to the sum of squares by sodium and phosphorus together is almost seventy times as great as the corresponding additional contribution by calcium.

The calcium and phosphorus correlations are not appreciably improved by the introduction of higher powers of these variates, but, as has been mentioned, there is evidence in sample 1 of a non-linear relationship between sodium content and succulence, the curve being concave to the water-content axis. Here, where the range of sodium content is greatest, the parabolic term is significant, having a coefficient of  $+0.558$  ( $P < 0.01$ ) after eliminating the linear term. But this gain is largely attributable to the phosphorus-content relationship with succulence, since the elimination of the quadratic term for sodium from the partial  $WP \cdot Na$  reduces the value from  $+0.446$  to  $+0.314$ , now well below significance level. It is again impossible to assess definitely the relative importance of the two effects, but the prevailing evidence indicates that in sample 1 the parabolic relationship on sodium alone gives a better fit than combined linear terms on sodium and phosphorus, the multiple coefficients being respectively  $0.911$  and  $0.896$ .

Further, the elimination of the linear term of calcium as well as the quadratic term of sodium from the total phosphorus-water content correlation at sample 1 leaves the coefficient unchanged ( $+0.313$ ). Hence the coefficients representing the phosphorus-water relation after eliminating all effects ascribable to sodium and calcium increase from sample 1 to sample 3, just as do the corresponding partials  $WCa \cdot NaP$  and  $WNa \cdot CaP$ . This general increase in value with age is not confined to the partials, but is also found in the total and

again in the multiple coefficients. The later the sample is taken, therefore, the better is the prediction achieved. This is probably due largely to the fact that the differences in water content between treatments increase progressively with age, the variance at sample 3 being about 2.6 times as great as at sample 1; if sampling errors remain constant their effect in obscuring the correlations will diminish with age.

Table II shows that the effects of sodium and phosphorus alone are statistically confirmed in the first two samples, while it has been pointed out that the calcium effect even in sample 3 is relatively slight. The regression equations on the first two variables, which give good prediction values in all samples, are as follows:

$$\begin{array}{ll} \text{Sample 1} & Y = 26.960 \text{ Na}_2\text{O} + 57.602 \text{ P}_2\text{O}_5 + 489.37 \\ \text{,, 2} & Y = 33.204 \text{ Na}_2\text{O} + 67.787 \text{ P}_2\text{O}_5 + 434.64 \\ \text{,, 3} & Y = 42.218 \text{ Na}_2\text{O} + 177.286 \text{ P}_2\text{O}_5 + 347.47 \end{array}$$

An upward trend of the regression coefficients with age, and a downward trend of the constant term, are very pronounced. When the calcium term, significant only for sample 3, is included, the others are but slightly affected:

$$\begin{array}{ll} \text{Sample 1} & Y = 28.486 \text{ Na}_2\text{O} + 7.535 \text{ CaO} + 56.791 \text{ P}_2\text{O}_5 + 470.71 \\ \text{,, 2} & Y = 37.959 \text{ Na}_2\text{O} + 20.501 \text{ CaO} + 54.501 \text{ P}_2\text{O}_5 + 397.13 \\ \text{,, 3} & Y = 49.407 \text{ Na}_2\text{O} + 31.617 \text{ CaO} + 156.853 \text{ P}_2\text{O}_5 + 299.90 \end{array}$$

The regression coefficient for calcium is seen also to increase considerably between samples 1 and 3, and its introduction into each equation raises the sodium coefficient, and lowers that of phosphorus together with the constant term. In sample 2, and also in sample 3, where the calcium effect is statistically significant and most clearly established, the calcium coefficient is seen to approach in magnitude that of sodium. It was mentioned above that the total effect of calcium at sample 3 is small compared with those of both sodium and phosphorus; this, then, appears not to be due to the fact that the effect of a given concentration of calcium within the plant is small compared with that of the same concentration of sodium, but that the variability of calcium content among the treatments was considerably less than that of sodium. The variance of sodium content at each sample was, in fact, more than ten times that of calcium. This is mainly accounted for in two ways: (1) all treatments received some calcium, though some received no sodium; and (2) calcium is less readily taken up than sodium, so that while excessive internal concentrations of sodium are found in some treatments, excessive concentrations of calcium are absent.

So far the relationships determining water content in the separate nutrient series of varying potassium level have not been considered. Treatment effects of the various elements may be subdivided into effects within and between the three levels of potassium supply, K<sub>1</sub>, K<sub>3</sub>, and K<sub>5</sub>. As between the levels there are only two degrees of freedom and therefore partial effects cannot be determined, but some attempt may be made to evaluate these effects within each of the three groups, although groups K<sub>1</sub> and K<sub>3</sub> comprise only six sets of data at



each sample. Here, again, variations in potassium content do not assist in elucidating the observed values of water content and the calcium effect may be neglected. The remaining two variates, sodium and phosphorus content, yield the partial regression coefficients shown in Table III.

TABLE III

*Partial Regression Coefficients within the Potassium Groups of Water Content (per cent. Dry Weight)*

(1) On per cent. sodium content, eliminating phosphorus content.

Sample.	K <sub>1</sub> .	K <sub>3</sub> .	K <sub>5</sub> .	Mean.
1	16.2	21.9	28.6	22.2
2	11.1	38.3	29.9	26.4
3	17.6	35.4	43.4	32.1
Mean	15.0	31.9	34.0	—

(2) On per cent. phosphorus content, eliminating sodium content.

Sample.	K <sub>1</sub> .	K <sub>3</sub> .	K <sub>5</sub> .	Mean.
1	-42.7	112.8	85.5	51.9
2	-10.8	33.6	97.5	40.1
3	63.8	124.9	167.7	118.8
Mean	3.4	90.4	116.9	—

In spite of the meagreness of the data for this purpose, there can be no doubt that a considerable rise in value of the coefficients occurs as the general level of potassium supply falls, the largest difference being found between K<sub>1</sub> and K<sub>3</sub>. This may, of course, be construed as an effect ascribable to potassium; indeed, between the three groups falling supply is associated with diminishing internal content, to which the increase in value of the regression coefficients is also related. High significance of the coefficients is found everywhere at the K<sub>5</sub> level, but in the K<sub>3</sub> group those of phosphorus content give a decidedly worse fit; while in the K<sub>1</sub> group only one significant value, for sodium, is found. Possible causes for these differences will be discussed later (p. 418).

Before leaving the question of the elucidation of treatment effects, a diagram of the general relationship established between sodium, phosphorus, and water contents may be presented (Fig. 1); for this purpose the sample totals have been used. Sodium content has been plotted against phosphorus content, and the correlation diagram for these two variates constructed, giving twenty-two treatment points, shown on the diagram as dots. Superimposed on the figure are the contour lines of water content derived from the equation:

$$Y = 34.815 \text{ Na}_2\text{O} + 94.685 \text{ P}_2\text{O}_5 + 415.24.$$

The positions of the dots referred to these lines thus give the water-content values of the various treatments derived from the equation. Each predicted value is associated with an observed value, shown by a cross displaced from the predicted value along the line of greatest steepness of the regression surface, and joined to it by a line whose length represents in terms of water

content the deviation from the regression equation of that particular treatment. The lengths of these lines can, of course, be further reduced significantly by allowing for calcium content.

2. *Relationships between sampling times.* Turning now to another aspect of

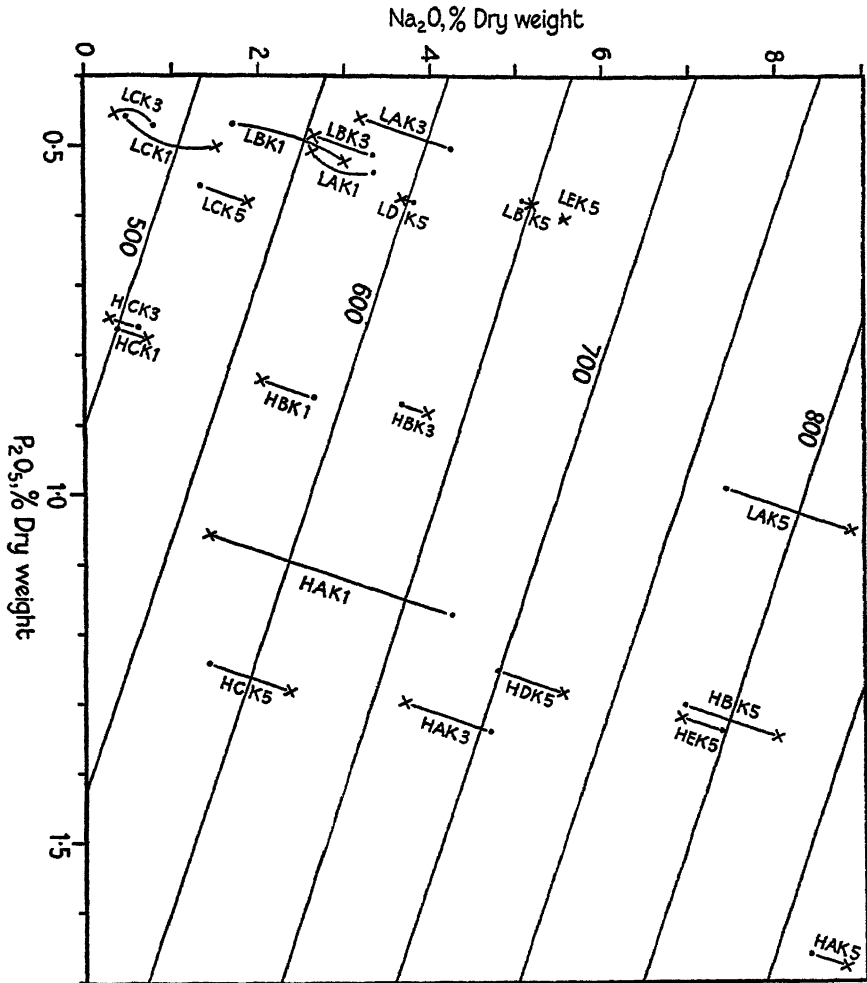


FIG. 1. Correlation surface of water content on sodium and phosphorus contents, derived from the sample totals. Dots indicate for the various treatments the water-content values given by the regression equation, while crosses indicate corresponding observed values.

the analysis of co-variance, i.e. relations between the treatment totals, it is clear that analysis here is of little value. Since there are only three samples it is impossible to obtain partial effects of the individual elements. The relevant primary data are given in Table IV, together with the formal total correlation coefficients. All variates fall with advancing sampling time. With sodium and

TABLE IV

*Totals of All Treatments (i.e. Totals of Columns in Table I)*

Sample.	H <sub>2</sub> O.	K <sub>2</sub> O.	Na <sub>2</sub> O.	CaO.	P <sub>2</sub> O <sub>5</sub> .
1	14.592	44.81	89.37	39.05	24.59
2	13.638	31.35	86.07	31.93	17.97
3	13.077	21.16	70.27	26.11	13.91

*Total Correlation Coefficients*

WK.	WNa.	WCa.	WP.	5%.	1%.
-0.997,6	-0.872,8	-0.995,9	-0.999,94	0.996,9	0.999,88

calcium the fall is not shown to be significantly related linearly to that of water content, though that of calcium nearly attains significance; the potassium relation exceeds its 5 per cent. point, while the phosphorus relation exceeds its 1 per cent. point. During an age drift the changes in a large number of possible variates are all closely correlated; hence experimentation over a wide range and with considerable precision is necessary to enable the immediate causes of the change in any one such variate to be distinguished. Clearly the present results supply no real information on the matter at issue. Moreover, it is possible that the correlation between potassium and water content obtained by James (1931) was due very largely to such secondary causes and not primarily to a direct relation between the variables.

3. *Relationships within the interaction of treatments with sampling times.* Correlations within the interaction provide results at first sight discordant with those described between treatments, seeing that here much the closest fit is obtained with potassium content, though again the total sodium and phosphorus correlations are positive and significant. If these last alone are considered, the partial regression coefficients are of the same order as are found between treatments, i.e. Na<sub>2</sub>O<sub>1</sub>+15.8; P<sub>2</sub>O<sub>5</sub>+117.6. The total correlation coefficients are as follows:

WK.	WNa.	WCa.	WP.
+0.746	+0.326	-0.174	+0.417

The potassium relation remains high after eliminating effects of the other elements, but, on the contrary, all other relations are reduced below significance level on eliminating potassium content, though that with sodium is still positive and nearly reaches 5 per cent. significance even after eliminating effects ascribable to all three other elements. It is not surprising that the three elements which gave good prediction of the water-content differences between treatments cannot be shown to exert effects in the interaction, since the magnitude of the latter is small compared with treatment differences; indeed, the *residual* mean square derived from the water-content sample totals after fitting constants to the three variables giving significant effects is twice as great as the *total* interaction mean square. Hence the apparent lack of effects here ascribable to sodium, calcium, and phosphorus is not inconsistent with the

large effects found between treatments, but at the same time the comparative small magnitude of the interaction only stresses the precision and possible importance of the effect ascribed to potassium, a relation which therefore warrants closer scrutiny.

The total interaction may conveniently be divided into two parts: (1) the general change of water content in each treatment from samples 1-3 as correlated with the corresponding change in potassium content; these may be measured simply as the differences for the individual treatments between the first and third samples; and (2) correlations over all treatments of the departures by the values observed at the second sample from this general change, conveniently measured as the mean of the values at samples 1 and 3 less the value at sample 2. The correlation coefficients within these two components between changes of water and potassium contents with time are  $+0.791$  and  $+0.221$  respectively. The former is highly significant and the latter negligible; hence the whole effect is found in the general changes in the above relation occurring between samples 1 and 3, irregularities at sample 2 being unrelated. The relationship, however, is not a simple linear one, since the quadratic term on changes in potassium content as related to changes in water content may also be shown to be significant after fitting the linear term. The maximum of the parabola of regression of change in water content on change in potassium content occurs within the range of observed changes in potassium content.

This portion of the interaction may be analysed further into effects within and between the three groups of potassium supply, i.e.  $K_1$ ,  $K_3$ , and  $K_5$ ; the range of change in potassium content within any one group does not overlap the range within any other. The results are again consistent with a roughly parabolic regression curve; the correlation coefficient within the  $K_5$  group is  $+0.770$  and is significant, that within the  $K_3$  group is negligible ( $-0.213$ ), while that within the  $K_1$  group, though negative and again fairly high ( $-0.673$ ) is not significant. But the greatest part of the variance is accounted for by the two degrees of freedom representing differences between the three groups; here the correlation coefficient is  $+0.999,22$ , and in spite of there being only three observational pairs this must be regarded as significant.

The relation of potassium and water content in the interaction between treatments and time is therefore not a simple one. At different levels of potassium supply there may apparently be a direct ( $K_5$ ) or an inverse relation ( $K_1$ ). Since at the higher levels there is no evidence of a direct relation, the cause cannot lie wholly in relations of osmotic pressure; for if these were at all considerable and were undisturbed by other factors then they should be greatest at the  $K_1$  level, where the largest variations in potassium content occur, and least at the  $K_5$  level. Moreover, the significant direct relation at the  $K_5$  level is unlikely to be explained in this manner, seeing that the absolute range of variability in potassium content is here very small, while in water content it is correspondingly large, leading to a regression coefficient of change in water content on change in potassium content of over 260. Almost the

whole of this significant correlation is due to the sub-grouping of phosphorus treatments within the  $K_5$  series. For the  $LK_5$  treatments consistently decrease in potassium content between samples 1 and 3 whereas the  $HK_5$  treatments consistently show small gains. With the exception of treatment A, all

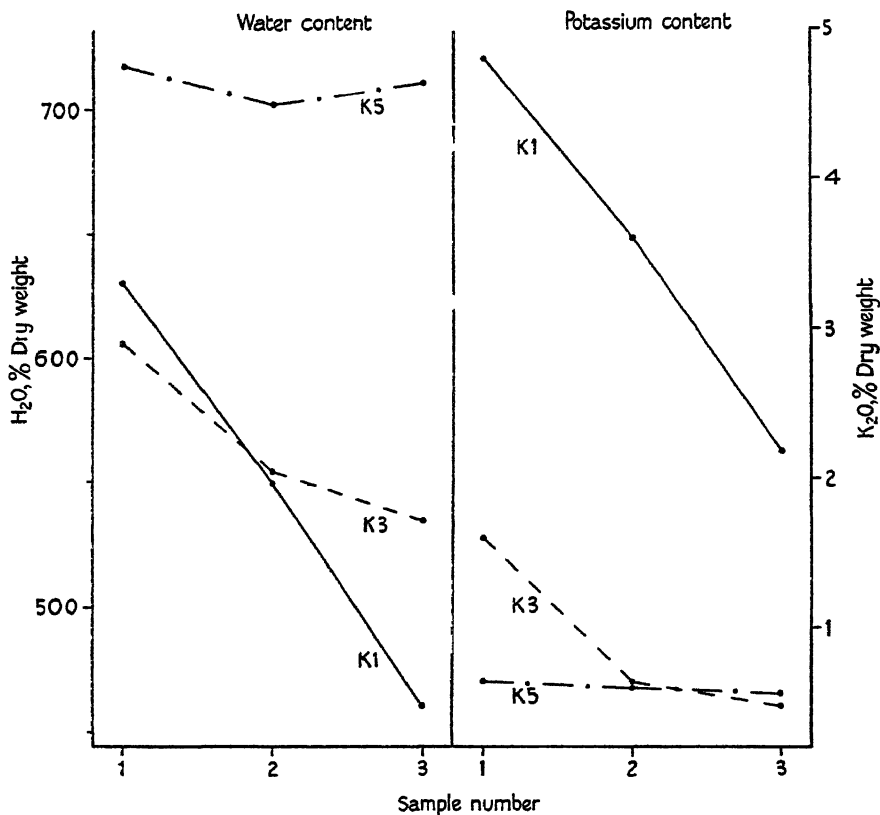


FIG. 2. Interaction between potassium level ( $K_1$ ,  $K_3$ , and  $K_5$ ) and sampling time of (1) water content, (2) potassium content. Points plotted represent the mean values of all treatments at one potassium level.  $K_1$ , maximum potassium;  $K_5$ , minimum potassium.

the  $HK_5$  treatments gained while the  $LK_5$  treatments as consistently lost in water content. Within neither of the phosphorus sub-groups is there evidence of a relationship between the changes in potassium and water contents. Reasons will be presented later (p. 418) for the view that the water content relationship between the phosphorus sub-groups is only indirectly related to potassium content.

The most important part of the interaction, as stated, is that between the three potassium levels, and is illustrated in Fig. 2. Here are plotted side by side against sample number the mean water contents and potassium contents of the three nutritional groups. A comparison of the relative positions

occupied in the diagram by the curves for water content and those for potassium content illustrates the generally inverse relationship between water and potassium contents among these groups. At each potassium level, however, the changes with time (forms of corresponding curves) are very similar in both variables; at the K<sub>1</sub> level a rapid decline of both water and potassium contents occurs, at the K<sub>3</sub> level a less rapid fall in both, while at the K<sub>5</sub> level no appreciable change in either variable is found. The question whether this relationship is a direct one will be taken up later (p. 417).

#### DISCUSSION

The primary result established in the preceding section is that the complex variations found in leaf water content by altering the level of potassium supply over a wide range of nutrient types can be very largely accounted for in terms of the contents in the plants of the various nutrient elements, potassium, sodium, calcium, and phosphorus; and doubtless more complete chemical analyses of further elements would yield still better prediction values. Potassium proves to be the only one of the four observed elements which, apart from small interaction effects, fails to show significant correlations with water content, hence hypotheses which assign to it a direct predominant role in succulence appear to be ill founded in fact. The reason for the apparent independence of water content and potassium content is not altogether clear, though it is probable that a large proportion of the total potassium within the cell is found in the cytoplasm; and possibly under conditions of shortage, practically all may be so situated. Since the element evidently plays a vital part in the life of the cell it appears possible that as regards succulence cytoplasmic changes occasioned by potassium may be at least as important as osmotic effects in the vacuole; if there is more than one effect these need not be in the same direction.

But if direct effects of potassium on succulence are difficult to establish, indirect effects are more evident, and these may be in either direction. Variations in potassium supply lead to a large number of other differences affecting succulence, and these are not confined to changes in content of the mineral elements as here examined. Nor do cytoplasmic changes of indefinite nature exhaust the indirect effects, for photosynthesis and growth rate are both profoundly influenced by potassium and the resulting variations in carbohydrate content certainly exert large effects on succulence. It is, perhaps, here that the incompleteness of the present data is most apparent, and some effort to assess the water-content differences due directly to carbohydrate is desirable in elucidating this experiment. When effects reasonably ascribable to carbohydrate are taken into account, a further possible explanation emerges of the apparent absence of direct osmotic effects of potassium.

High carbohydrate plants are characterized by low succulence; moreover, through the life-history of one plant a marked negative correlation is found between succulence and carbohydrate content. This general inverse relation must itself be due to a variety of causes. In the first place, the high

carbohydrate plant lays down heavier walls, which fact immediately reduces water content by increasing dry matter without appreciably affecting the absolute quantity of water. Insoluble carbohydrate fractions, such as starch, where they occur, act in precisely the same way. Apparently opposed to these effects is the osmotic activity of the soluble carbohydrate fractions. But it must not be assumed that an increase of the osmotic pressure of the cell sap, due to the gain of solutes from external sources, necessarily leads to increased water content. A high carbohydrate leaf of the barley type may contain soluble sugars to the extent of 4–5 per cent. of the fresh weight, or, say, 25–30 per cent. of the dry weight. Let a hypothetical leaf have originally 25 per cent. soluble sugar and let this sugar be itself increased by 20 per cent. The immediate effect is to increase the sugar content to 28.6 per cent. of the dry weight while the dry weight itself increases by 5 per cent. Unless the weight of water within the leaf similarly increases by 5 per cent. the final result of the increased sugar will be a reduction of water content. In order merely to maintain the original water content, therefore, the cell volume needs to increase by approximately 5 per cent. The force tending to take in water is that due to the extra osmotic pressure of the additional sugar, which in this case is roughly one-fifth of the original osmotic pressure due to sugar alone, and so considerably less than one-fifth of the total original pressure, even if the whole of the sugar is in the sap. The cells being already assumed turgid and the walls stretched, it appears probable<sup>1</sup> that a very slight increase in volume will be sufficient to reduce to zero the suction pressure and that the final result of the increased sugar content may thus be a decreased water content. This is particularly the case in the high carbohydrate leaf, which, as has been mentioned, has thicker walls, and extensibility must decrease rapidly with increasing thickness. Probably the most important of the carbohydrate effects to be anticipated from the present point of view is this effect on wall extensibility, so that with increasing carbohydrate level the change of water content relative to a given change of suction pressure will decrease.

In dealing with the change in amount of any osmotically active substance in the sap other than sugar, and provided the change is a true gain or loss of the substance by the leaf and not one among the leaf constituents themselves, from the point of view of water content there are always the two opposed effects to consider: change in total water consequent on osmotic changes, and change in total dry weight. The slope of the curve connecting water content with weight of the active substance therefore depends on the weight of the osmotically active unit in the substance, a light unit resulting in a large positive slope, while a very heavy unit may even have a negative slope. These various slopes are also all dependent on wall extensibility, and hence on carbohydrate level of the leaf, falling towards zero as the walls thicken and harden.

Somewhat similar considerations apply not only to substances which may

<sup>1</sup> Thus Bennet-Clark *et alia* (1936) estimate the turgid volumes of cells of certain root and petiolar tissues to be probably less than 5 per cent greater than the flaccid volumes.

exert osmotic pressure in the sap, but also to any which may accumulate in the cytoplasm and by their accumulation increase its degree of hydration. Water content increases of this nature are minimized by the increase in dry weight; while water content decreases arising from the accumulation of substances lowering the degree of hydration, are exaggerated by the same cause. From the point of view of succulence the two aqueous systems, cytoplasm and sap, tend to buffer one another. If, for instance, a change occurs which decreases the vapour pressure of the cytoplasm, the latter will take up water. Water entering from outside results in cell expansion and increase of turgor pressure; but throughout the cell pressure is uniform, therefore for equilibrium the vapour pressure of the sap must also decrease, i.e. osmotic pressure must increase. The net result of the change is that a not inconsiderable part of the water taken up by the cytoplasm is drawn from the vacuole, which actually diminishes in size as the total cell-volume increases. Should there be large secretion pressures such as have been envisaged by Bennet-Clark *et alia* (1936) this buffering action will be unaffected provided the secretion pressure does not itself change with the amount of water in the cytoplasm.

In this experiment carbohydrate determinations were not made, though estimates of net assimilation rates are given by Shih (1936). But a very good notion may be obtained of the general trend of total sugar content in the twelve treatments comprising series A and C from a comparison with the entire-leaf data given by Russell (1938). In his experiment the same treatments were studied, although in another season. The data from sample 2 of his experiment are known to be unreliable owing to its coincidence with a storm of abnormal severity, but those from samples 1 and 3 may be compared with the corresponding samples of this experiment. Russell's data are given on a fresh weight basis, but the present water content figures may be used to reduce them approximately to terms of dry weight. For the present purpose the basis is largely immaterial, since both lead to substantially the same result.

By dividing these data into groups according to the level of potassium supply it is clear that while at both samples the mean total sugar content decreases considerably as potassium supply is reduced, at sample 1 the difference between the groups is much less pronounced than at sample 3. In the K<sub>1</sub> and K<sub>3</sub> series total sugar content increases rapidly between samples 1 and 3, but in the K<sub>5</sub> group the content shows only a small change with age, the tendency being to decrease.

It will be observed that this interaction effect between age and level of potassium supply on sugar content is precisely the inverse of the corresponding interactions in both water content and potassium content as given in Fig. 2. Moreover, in discussing these latter it was pointed out that while water content and potassium content were closely related in the interaction, the relative general levels of the three curves in the diagrams was very different. No such radical difference occurs between sugar content and water content, hence it appears highly probable that the main relation between water and potassium



found in the interaction is spurious and would disappear if effects ascribable to carbohydrate could be eliminated from the correlations.

The other high positive correlation in the interaction, that within the K<sub>5</sub> group, appears to be likewise spurious. As was shown earlier (p. 414), it is a correlation existing between the phosphorus level sub-groups, HK<sub>5</sub> and LK<sub>5</sub>. The difference between these sub-groups in change of potassium content with time is certainly due to the much greater growth rate of the LK<sub>5</sub> group than the HK<sub>5</sub>; while the difference in change of water content is consistent with a probable difference in change of carbohydrate, deduced from Russell's data. The argument for this proceeds along precisely similar lines to those shortly to be put forward (p. 420) in explaining the increase of the phosphorus regression coefficient with age, and need not be anticipated here.

The only other effect which emerged from the experiment as possibly ascribable directly to potassium was the increase of the regression coefficients within the potassium groups as we pass from K<sub>1</sub> to K<sub>5</sub> (Table III), and it appears likely that this phenomenon too is explicable in terms of carbohydrate. In the first place it may be stressed that the differences between the coefficients from the potassium groups appear to be real. The largest difference occurs between K<sub>1</sub> and K<sub>3</sub>, and this cannot be explained by greater variation among the six treatments of K<sub>3</sub> than among those of K<sub>1</sub> in the contents of sodium and phosphorus, with consequent more accurate evaluation; for the variances within the two groups are almost identical in the first two samples, becoming greater in K<sub>3</sub> only at sample 3. At the same time the variance of water content is over six times as great in K<sub>3</sub> as in K<sub>1</sub> at sample 1, and the discrepancy is greater still at sample 2. In other words, while at the K<sub>3</sub> level the leaves respond readily to changes in mineral content by concomitant changes in succulence, at the K<sub>1</sub> level they are unresponsive; at the K<sub>5</sub> level slightly greater responsiveness still is apparently found. Differences of this kind are to be expected if carbohydrate level falls from K<sub>1</sub> to K<sub>5</sub>.

At the same time it appears probable that the considerable rise of the regression coefficient from K<sub>1</sub> to K<sub>3</sub>, and the comparatively small change from K<sub>3</sub> to K<sub>5</sub>, do not exactly reproduce the mean differences of carbohydrate level between these groups; as regards sugar content, K<sub>3</sub> is much more symmetrically placed between K<sub>1</sub> and K<sub>5</sub>. While this difference might be accounted for by a strongly curved relation between sugar content and cell-wall extensibility, there are certainly errors in the estimates of the regression coefficients due to grouping which might equally account for it, i.e. while level of potassium supply and sugar content are fairly highly correlated over all treatments there are marked exceptions, and grouping according to level of potassium supply is not identical with grouping according to sugar content. The most anomalous treatments from this point of view are those designated CK<sub>3</sub>; in general, these have higher sugar contents than corresponding CK<sub>1</sub> treatments and much higher contents than corresponding AK<sub>3</sub> treatments. It is to be noted that they likewise have *reduced* water contents relative to their

K<sub>1</sub> counterparts, a fact which in the past has been explained as a direct result of reduced potassium content. This difference between the A and C types does not increase from K<sub>3</sub> to K<sub>5</sub>, but tends to diminish.

Also the C treatments have minimal, and the A treatments maximal, sodium contents; the same difference holds for phosphorus content. By considering

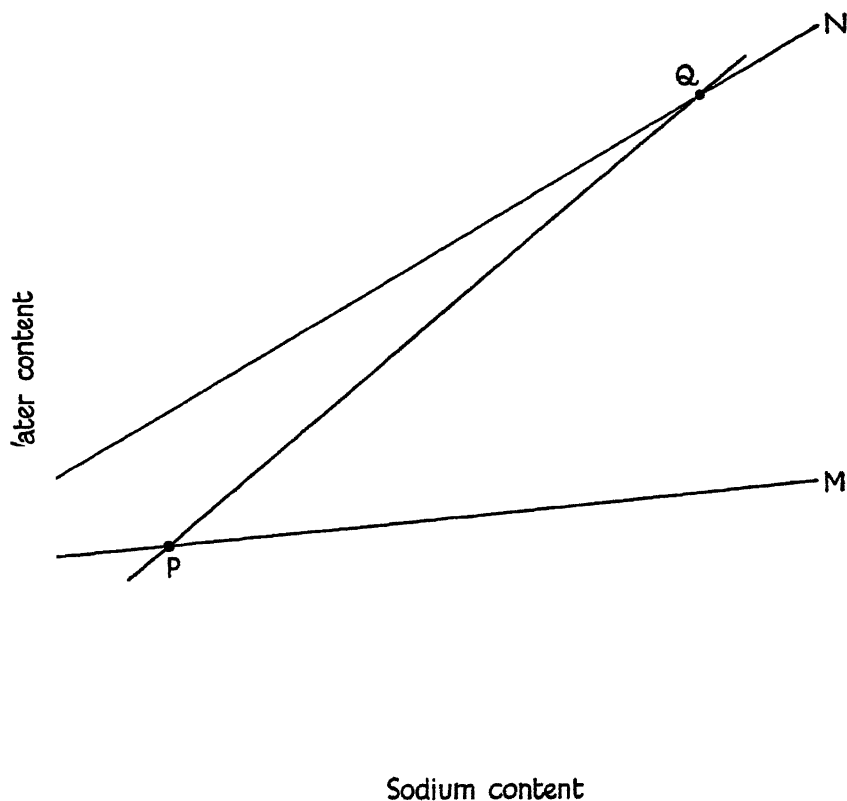


FIG. 3. Effect of treatment grouping on observed relation between water content and salt accumulation, when differences of carbohydrate level are involved in the grouping. M, high carbohydrate relation; N, low carbohydrate relation. For full explanation, see text.

K<sub>3</sub> as one group, therefore, and correlating water content with sodium and phosphorus, neglecting differences of carbohydrate within the group, selection is introduced in such a manner as to over-estimate the regression coefficients. For the relation between water content and sodium content may be represented by a series of curves differing in height and slope according to carbohydrate level, somewhat as shown in Fig. 3. Curve M gives the relation with a high carbohydrate type of leaf, corresponding to CK<sub>3</sub> treatments, and N the relation with a low carbohydrate type, corresponding to AK<sub>3</sub>. Curve N is likely to lie everywhere above M, since at zero sodium content there remains

the osmotic pressure due to other substances, whose total amounts are assumed to be roughly equal; with no sodium these give water contents related inversely to carbohydrate level. Point P gives the sodium-water content relation of the C type, and Q that of the A type. The observed regression is therefore the line PQ, which is steeper than the true regression at either carbohydrate level. Even if N were below M at zero sodium content and the curves intersected at some particular sodium content, if we assume the three regressions are straight the slope of PQ will always be greater than the mean slope of M and N provided the positive difference of water content between N and M at the sodium level of Q is numerically greater than the negative difference at the sodium level of P. It is therefore reasonably certain that in the experiment the coefficient within the K<sub>3</sub> group is in fact over-estimated. Since differences of this kind between the A and C types are less pronounced in the K<sub>5</sub> group than the K<sub>3</sub>, while the mean carbohydrate level is lower, it appears probable that the differences in the observed regression coefficients derived from the three potassium groups are very largely accountable in terms of carbohydrate differences.

The general increase of the regression coefficients between samples 1 and 3 is also probably accountable on the same hypothesis. No attempt will be made to deal with this aspect in any detail, indeed without further knowledge of the actual carbohydrate fractions this is impossible; but the probable main causes of the increasing *total* regression coefficients at least may be briefly outlined. The greatest increase with age is found with phosphorus. The total correlation between phosphorus and water contents is fairly high, e.g., for the sample totals, +0.743. A large part of this correlation must arise from the water content and phosphorus content differences of the two series H and L; except at sample 3, only one treatment from the L series, namely, LAK<sub>5</sub>, has a phosphorus content within the range of variability of the H series. Russell's figures show that the increase in total sugar content between samples 1 and 3 is considerable in the L series and very much less in the H<sup>1</sup> series, so that while at sample 1 the H series is at a rather higher sugar level than the L series, the position is decisively reversed at sample 3. Also, the average fall in contents of potassium, sodium, and calcium is almost identical in the two groups. Responsiveness to phosphorus content of the treatments which have higher phosphorus and water contents may be expected therefore to change little with age, and that of the treatments with lower phosphorus and water contents to decrease; moreover, the increase in carbohydrate level of the L series will lower the water content here irrespective of phosphorus content. The net result is an increase with age of the slope of the regression line of water on phosphorus content, and a considerably greater decrease of water content in the L series than the H series, an effect which was statistically established in the first part of the paper (Richards and Shih, 1940).

<sup>1</sup> Within the K<sub>5</sub> group this needs modifying. The same general difference between H and L holds, but in HK<sub>5</sub> a considerable drop occurred and in LK<sub>5</sub> a slight rise.

The explanation of the increase of the sodium coefficient is a little more complex, since the correlation diagram between water and sodium contents leads to a less simple grouping of the treatments along the regression line. At sample 1 the lower end of the curve comprises the six C treatments, in which both the variables, water content and sodium content, are minimal, while the upper portion of the curve is occupied solely by the K5 levels of all treatments receiving appreciable quantities of sodium. As salt concentration falls with age in the latter, carbohydrate also falls somewhat; therefore the positions of the points representing these treatments do not fall lower on the regression line but tend to move away more nearly parallel to the sodium axis. At the lower end of the curve are the C treatments; here carbohydrate increase with age is not very great, but the general fall in salt content is not paralleled by the fall in sodium content, since even at sample 1 sodium content is very low, none having been given in the nutrient solutions. Points at this end of the curve in later samples therefore tend to move away from the regression line nearly parallel to the water-content axis. These tendencies at the two ends of the curve amount to a rotation of the total regression line, increasing its steepness. The increase in the total coefficient is therefore due partly to measured variables, and this part is eliminated in the partial coefficient, in which the rise with age is in fact much less pronounced than it is in the total coefficient. The rise of the partial coefficient on sodium is also considerably less than that on phosphorus, as might be anticipated from the fact that there is less carbohydrate change here within the dominant groupings.

In the case of calcium content the increase of the regression coefficient between sample 1 and sample 3 is not easily interpreted, since the regression of water content becomes positive only after elimination of effects ascribable to sodium. It will not, therefore, be further considered.

From the foregoing discussion it is seen that the main difficulties in interpreting the statistical analysis may be largely accounted for in terms of effects reasonably ascribable to carbohydrate. The question immediately arises why it is possible to account so well for water-content differences in terms of the measured variables when an unmeasured variation in carbohydrate over the experiment is likely to have such far-reaching effects. The reason is doubtless that carbohydrate level is not distributed at random over the experiment, but may itself be very largely determined by simple regressions on the analysed fractions. Hence its general effects on succulence appear as modifications of the correlation and regression coefficients which would hold if carbohydrate content were uniform over the experiment and other factors remained unchanged. At the same time it is unlikely that all carbohydrate effects will be accounted for in such ways, and after fitting constants to the measured variables residual effects would remain not wholly distributed at random. The same will apply, of course, to any other unmeasured and non-random factors which affect water content appreciably. Such systematic errors in the predicted values of water content, after fitting constants to sodium, calcium, and

phosphorus contents, are indeed considerable, as is shown by the following correlation coefficients between the differences of observed and predicted values among the three samples:

Samples.	1 and 2.	2 and 3.	1 and 3.
Correlation coefficient	-0.724	+0.676	+0.505

All the correlations are highly significant. Potassium content does not, of course, account for them. While no two values differ significantly from one another, sample 2 is considerably more highly correlated with both 1 and 3 than are the latter pair together; hence it appears probable that the unmeasured factors affecting water content are changing continuously and differentially with age.

Having regard to the above discussion, a hypothesis which may explain the apparent independence of water content on potassium content throughout the experiment may now be advanced. Potassium was applied at three levels, but the internal contents of the plants fall naturally into two groups, K<sub>1</sub> with high contents, and K<sub>3</sub> and K<sub>5</sub> with very low and approximately equal contents. Within the K<sub>1</sub> group the highest potassium contents are comparable in magnitude with the highest sodium contents, although the variability is considerably less. It is quite probable that here potassium is exerting considerable pressures, but that appreciable effects on water content are not found because of the stability of the group, due to high carbohydrate level, already discussed. If sodium and phosphorus cannot be shown to affect water content here, there is no reason to suppose that potassium should be more favourably placed.

Within the deficient groups K<sub>3</sub> and K<sub>5</sub> the variance of potassium content is so small as to be negligible: thus at sample 1 the variance of potassium content is twenty-five times as great in K<sub>1</sub> as in K<sub>3</sub>, at sample 2 ninety-seven times, and at sample 3 one hundred and forty times as great. Similarly, at sample 1 the variance of sodium content in the K<sub>3</sub> group is ninety times that of potassium content, while at sample 3 it is over three hundred times as great. It is clearly unavailing to look for differential effects of potassium content within the groups K<sub>3</sub> and K<sub>5</sub>, while if there is a general effect it must also be so slight as to be inappreciable, since the contents of potassium are likewise of a very low order. Potassium differences of considerable magnitude are found only within the group K<sub>1</sub>, and between this group and the others, and it is probable that here their direct effects are obscured by the large superimposed carbohydrate effects. To discover direct potassium effects on succulence it would be necessary to diminish the correlation usually found between potassium and carbohydrate contents, and to study a plant displaying high potassium content together with thin cell-walls. Experiments with reduced light might provide the requisite conditions.

The decrease in water content with reduced potassium supply, often reported in the literature, appears to be in the main a consequence of increased

carbohydrate level, direct effects of potassium content being subordinate in magnitude. In these instances the basal nutrients supplied belong to a type much closer to the C series of this experiment than to the A series. Janssen and Bartholomew (1929, 1930) observed in this connexion increasing carbohydrate content accompanying the reduction in water content with potassium deficiency. Nightingale *et alia* (1930) have described potassium deficient tomato plants, grown in solutions containing calcium with a little sodium, in which supernormal carbohydrate contents accompanied reduced succulence in early stages of growth; later these symptoms were reversed, subnormal carbohydrate contents being associated with increased succulence. The sequence is similar to that in some of the present treatments at the K<sub>3</sub> level, and provides strong evidence of the importance of carbohydrate in this connexion. The culture solution used by Warne (1936) was also of the high calcium type, hence the explanation of his results is likely to be similar. With reduced potassium supply thicker walls were presumably laid down, and their rigidity resulted in a lower water content for a given turgor pressure. Hence the reduction he observed in cell-size was a secondary effect of potassium involving interactions with other ions and acting through change in carbohydrate level.

With regard to the individual effects ascribable to the other elements studied very little can profitably be said. For reasons already given, the value of the regression coefficient for any one is not unique, nor does its value at any one time represent the ideal value to be expected at any one carbohydrate level; neither are the relative values of the coefficients of the various elements at any one sampling time necessarily comparable, since, owing to effects of grouping, carbohydrate and other unknown factors affect these coefficients differentially. Moreover, the experiment cannot discriminate between effects which are osmotic in origin and those confined to the cytoplasm. On general grounds, provided carbohydrate level is not too high, correlations between succulence and an osmotically active substance must be positive, while correlations with a substance affecting cytoplasmic hydration may be either positive or negative. All correlations observed in this work, after eliminating effects of other elements, appear to be positive. Some measure associated with proteins might be useful in a further analysis, and possibly part of the relation with phosphorus content belongs here; phosphate might give both cytoplasmic and osmotic effects. Undoubtedly in this experiment effects of carbohydrate level are superimposed on the more direct phosphorus effects, and at the time of sample 3, when large sugar content differences are found between the high and low phosphorus series, such effects must be very considerable. This carbohydrate change probably explains completely the high value of the correlation coefficient, as well as the regression coefficient, between phosphorus and water contents in this sample.

The effect attributed to sodium is outstanding in the experiment. It is possible that it, too, is over-estimated, since sodium content appears to be

inversely correlated to an appreciable extent with carbohydrate level. The analysis of the whole experiment, of which only a part is concerned in this paper, reveals very distinct toxic effects of sodium; hence its effects are not confined to the vacuole, and the relationship with water content may not be entirely osmotic. Later experiments at this Institute confirm the large effects which sodium has on succulence. Thus, with a nutrient solution containing ammonium salts, only little calcium, and no basal sodium, the addition of a very small amount of sodium is found immediately to increase succulence.

The effect of calcium is perhaps unexpected. For high calcium plants are 'hard' and low in water content; a negative correlation with succulence might thus reasonably be expected, and indeed all total correlations are negative, often significantly so. But this proves to be a secondary effect due to the fact that when calcium is in excess uptake of other ions is reduced, while at the same time calcium itself is not accumulated in very large amounts. Hence the total internal salt content is low and with it water content. But the partial correlation of succulence on calcium content is positive, and the regression coefficient appears to be of the same order as that of sodium. Moreover, a considerable part of the total calcium is immobilized in the plant and removed from systems actively affecting water content; hence the magnitude of the regression coefficient on the effective calcium content would presumably be higher than that found here.

#### SUMMARY

1. From the data of an experiment in which barley was grown under twenty-two combinations of nutrients and sampled on three occasions during vegetative growth, an attempt is made by statistical methods to account for the observed differences in leaf water content in terms of the contents of certain analysed ash fractions, viz.  $K_2O$ ,  $Na_2O$ ,  $CaO$ , and  $P_2O_5$ .

2. The predominant effect in the experiment must be ascribed to sodium, large positive correlations, both total and partial, being found between sodium and water contents among the treatments at all sampling times. Highly significant positive correlations between phosphorus and water contents are also found everywhere. Sodium and phosphorus contents together account for a very large part of the total water content variance, the relations between the three variables for the sample totals being shown in Fig. 1 (p. 411).

3. Total correlations between calcium and water content are negative, but the elimination of disturbing effects ascribable to sodium reverses their sign, and significant positive effects are sometimes found.

4. *Potassium is the only element of those whose effects are measured which shows no appreciable relationship to succulence* between treatments, though in the interaction of treatments with times of sampling small and complex but highly significant relationships are found.

5. Partial regression coefficients of water content on both sodium and phosphorus contents are shown in general to increase markedly with reduction

in potassium supply, and also at any one potassium level to increase with the age of the plant.

6. Probable effects on succulence of variations in carbohydrate level among the various treatments, and also with age of the plant, are fully discussed. They are held to account for the apparent facts that water content becomes more sensitive to internal concentrations of the elements with (1) decreasing potassium supply, and (2) increasing age. It is concluded that the greatest effect of carbohydrate on succulence is that exerted on cell-wall extensibility as determined by thickness of wall; for a given suction pressure a thin-walled cell will absorb more water than a thick-walled, more rigid cell.

7. An hypothesis along similar lines is advanced to account for the absence of observed effects of potassium on water content.

In conclusion, the authors desire to express their gratitude to Sir John Russell for granting facilities for work at Rothamsted, to Professor V. H. Blackman for his ready interest, and especially to Professor F. G. Gregory, to whose inspiration the work owes much.

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# Study of the Somatic Chromosomes in *Narcissus*<sup>1</sup>

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With sixty Figures in the Text

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## I. INTRODUCTION

THE genus *Narcissus*, consisting of nearly thirty-five species (Engler and Gilg, 1924) most of which show polyploidy, forms an attractive group for cytological investigations. Although almost all the species of *Narcissus* possess fairly long chromosomes like other monocotyledonous plants, this genus has so far received little attention from cytological workers. Stomps (1919) was the first to give an account of chromosome numbers of some species. His investigations were followed by those of de Mol (1922, 1925, 1926, 1927) and Heitz (1926), Nagao (1929, 1930), and Fernandes (1931), who all added to the previous list of chromosome numbers in this genus. Nagao's (1933) account of meiosis in several species is also a valuable contribution. More recently Fernandes (1935, 1936) and Sato (1938) have studied the morphology of somatic chromosomes of several species and given their ideograms.

The study of the somatic chromosomes is instructive in several ways. It brings to light not only the numerical variation (polyploidy and aneuploidy) but also indicates the nature of evolution of chromosome complement in allied species and genera. The size of chromosomes in conjunction with the

<sup>1</sup> Part II of thesis approved for the Degree of Doctor of Philosophy of the University of London.

position of primary and secondary constrictions and the satellites or trabants indicates the chromosome type in a species, and any changes observed in these leads to the inference of translocation, fusion, or fragmentation, according to the changes involved. The studies of karyotype alteration on these lines in several plant genera, e.g. Liliaceae (Delaunay, 1926; Sato, 1936, 1937), Amaryllidaceae (Sato, 1938), Paeonia (Sinoto, 1938), Crepidinae (Babcock *et al.*, 1937), Allium (Levan, 1935), have already furnished interesting results.

With the discovery of satellites in plant and animal cells their study has become of increasing interest during the past few years. Gates (1938) has shown how the study of satellites and nucleoli can be brought to bear on the phylogeny of the nucleus, and a variation in their number correlated with auto- or allo-polyploidy. These data, when supplemented with other evidence, can form a reliable guide to the true basic number for the genus. Thus, species which appeared to be diploid from their chromosome numbers have been proved to be secondarily balanced polyploids. The most remarkable examples of this are afforded by *Oryza* (Nandi, 1936; Ramanujam, 1938) and *Cicer* (Iyengar, 1939).

With the discoveries of Heitz (1931) and McClintock (1934) a new field of inquiry has been opened in the study of the nucleolus. Although their work has solved some of the mysteries linked up with the organization of the nucleolus in plant and animal cells, there still remain a number of problems which require to be further studied. For example, the nature of the material from which the nucleoli are organized is not known with certainty. Again, there is very little evidence to show whether the nucleolar material is contributed by all the chromosomes in the complement or if it is elaborated only at a particular locus on the SAT-chromosomes. The exact nature of the factors controlling the size of nucleolus is also not understood. Moreover, some of the incongruities which have recently arisen with regard to the relation of secondary constrictions to the nucleolus also require to be explained.

It was with the object of furnishing evidence on some of these problems that the present study of *Narcissus* was undertaken.

## 2. MATERIAL AND METHODS

The following ten varieties belonging to six different species of *Narcissus* were under study. (1) *N. odoratus* (Mediterranean region), (2) *N. gracilis*, (3) *N. tazetta* (Mediterranean region), (4) *N. tazetta* var. *grand soleil d'or*, (5) *N. tazetta* var. *canaliculatus*, (6) *N. pseudonarcissus* var. *Victoria*, (7) *N. pseudonarcissus* var. *Emperor*, (8) *N. pseudonarcissus* var. *King Alfred*, (9) *N. poeticus*, (10) *N. incomparabilis* var. *Sir Watkins*. The cytological material of (1) to (3) was obtained from bulbs growing at Royal Botanic Gardens, Kew, and I take this opportunity of thanking the authorities for allowing me to collect the root-tips.

Bulbs of varieties (4) to (10) were obtained from Messrs. Barr & Sons,

London, and planted during the month of October, 1938, in pots containing moist sand, which were placed inside the hothouse of the Botany Department, King's College, London. The root-tips were ready for fixation about a week after the bulbs were planted.

Root-tips were fixed in Benda's solution, La Cour's 2 BE, Navashin's fluid, and chromic formalin (1:1). The first two fixatives provided excellent metaphase plates in which the constrictions of chromosomes were very clear. For observations on metaphases, transverse sections of root-tips were cut  $16\mu$  thick and stained by iodine-gentian violet method.

Navashin's fluid proved suitable for observing the attachment of SAT-chromosomes to the nucleolus at prophase, as well as for observations on organization of nucleolus in telophase. For both these purposes longitudinal

TABLE I

Showing the Number of Chromosomes, Satellites and Nucleoli, etc. in the Somatic Complement of Different Varieties of *Narcissus*.

Serial No.	Name of species.	Name of variety.	Chromosome number (2n).	Number of				Reference to figures.
				SAT-chromosomes.	Secondary constrictions.	Nucleoli in telophase.	Chromosomes attached to the nucleolus at prophase.	
1.	<i>N. odorus</i>	—	14	2	—	2	2	1, 3, 4
2.	<i>N. poeticus</i>	—	14	2	2	2	2	6, 7, 8
3.	<i>N. gracilis</i>	—	14	2	2	2	2	10, 11, 12, 13
4.	<i>N. tazetta</i>	<i>Grand soleil d'or</i>	20	2	6	2	2	16, 18, 21
5.	" *	—	21	4	—	4	—	27, 28, 29
6.	"	<i>canaliculatus</i>	29	4	2	6	—	30, 31, 32
7.	<i>N. pseudonarcissus</i>	<i>King Alfred</i>	28	4	—	4	4	43, 44, 45, 48
8.	"	<i>Emperor</i>	21	—	—	3	3	55, 59
9.	"	<i>Victoria</i>	22	3	2	3	3	34, 35, 36, 40
10.	<i>N. incomparabilis</i>	<i>Sir Watkins</i>	21	3	—	3	—	49, 50, 54

\* This is an unnamed variety from the Mediterranean region.

sections of root-tips were cut at a thickness of 10 to  $12\mu$ , and stained according to Feulgen-light-green technique (Semmens and Bhaduri, 1939). The chief difficulty experienced in the earlier attempts with Feulgen staining was the diffusion of fuchsin colour throughout the nucleus, which hindered critical examination of telophases. This difficulty was surmounted by hydrolysing the slides for about fifteen minutes in a weak acid solution (5 c.c. HCl: 150 c.c. distilled water). This modification was, however, not used for seeing the prophase attachment, in which case slides were hydrolysed in N/HCl for twenty to twenty-five minutes.

Chromic formalin, as such, did not prove useful for nucleolus studies, as brightly stained nucleoli could not be obtained with it. However, by pre-mordanting the slides overnight in 5 per cent. aqueous acetic acid their staining was considerably improved. Therefore, if light green is to be used as a

counterstain for the nucleoli, the presence of acetic acid in the fixative seems absolutely necessary for *Narcissus*.

### 3. CYTOLOGICAL OBSERVATIONS

#### (a) *Somatic Chromosomes*

##### (A) *N. odorus* ( $2n = 14$ ).

Fernandes (1931) reported the chromosome number of this species as  $2n = 10$ , but Nagao (1933) has reported  $2n = 14$ . The present observations agree with those of Nagao (Fig. 1). The somatic complement of this species can be classified as follows (see ideogram, Fig. 5):

(1) One longest pair (AA) with sub-median primary constrictions. This pair, although of the same length, differs slightly in the position of the spindle attachment region. The proximal arm of one chromosome was found to be always longer than that of its mate.

(2) Two pairs (BB and CC) long with sub-terminal constrictions. One of the C chromosomes bears a satellite on the proximal end.

(3) One pair long (DD) with sub-median constrictions, one of the D chromosomes bearing a satellite on the longer arm.

(4) Two pairs of medium length (EE and FF) with sub-median constrictions.

(5) One pair (GG) small with sub-terminal constrictions. This pair is also heteromorphic.

Fig. 3 shows the attachment of the two SAT-chromosomes to the nucleolus at prophase. From this figure also it can be seen that the SAT-pair is heteromorphic. Fig. 4 is telophase showing two nucleoli in each daughter nucleus, corresponding to one pair of satellited chromosomes.

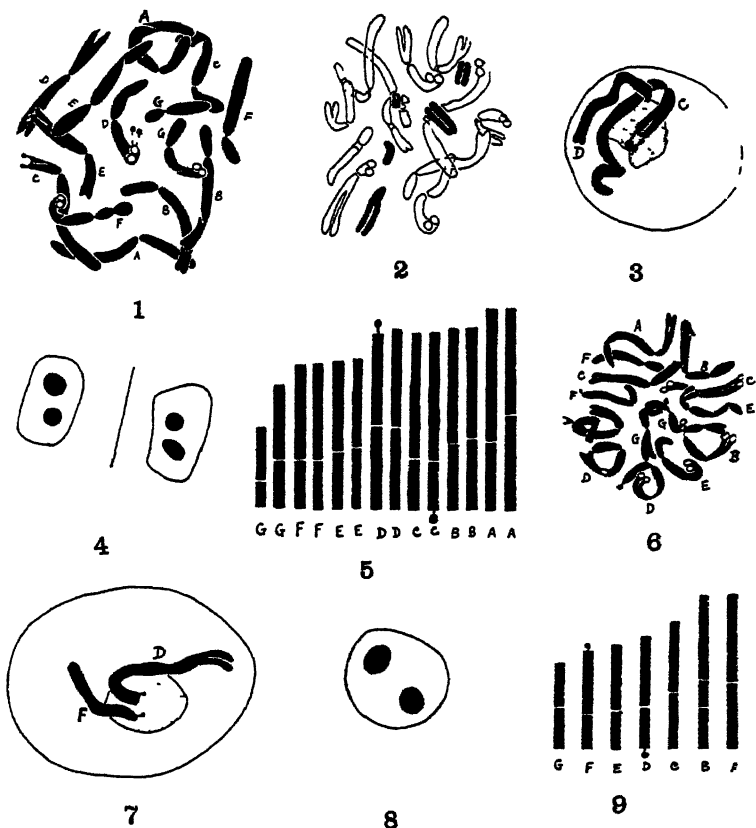
The heteromorphic nature of certain pairs of chromosomes, including the SAT-pair in this species, is quite consistent with the view that it is of hybrid origin. Some taxonomists regard it as a hybrid between *N. Jonquilla* and *N. pseudonarcissus*.

##### (B) *N. poeticus* ( $2n = 14$ ).

The count of fourteen chromosomes in this species (Fig. 6) agrees with that reported by de Mol (1928). The somatic complement consists of (see ideogram, Fig. 9):

(1) Two pairs long (AA, BB) with sub-median primary constrictions. The pair BB has also a median secondary constriction on the short arm.

(2) Four pairs (CC, DD, EE, FF) of medium length, the size of these decreasing in order. Pairs CC and DD have nearly sub-median primary constrictions, whereas pairs EE and FF have sub-terminal constrictions. One of the D and one of the F chromosomes are satellited, the satellite in the former being on the proximal end, whereas in the latter it is borne on the distal end.



All figures were drawn at the bench-level with the aid of camera-lucida using Beck's 2 mm. apochromatic objective N.A. 1.2 and Beck's compensating eye-pieces 25 and 17, giving an approximate magnification of 3,350 and 2,450 diameters, respectively. Figures 4, 21, 29, 38, 39, 40, 41, 42, 46, 47, 52, 53, 54, 58, 59 and 60 have been drawn with the eye-piece 17 and all the rest with 25. The figures have been reduced one-third in reproduction.

FIGS. 1-9. *N. odorus*. Fig. 1. Somatic plate with fourteen chromosomes, one heteromorphic pair CD being satellited. Fig. 2. Somatic plate with twelve chromosomes and five fragments. Fig. 3. Prophase nucleus showing the heteromorphic pair of satellites (CD) attached to the nucleolus. Fig. 4. Telophase showing the nucleoli in each daughter nucleus. Fig. 5. Diagrammatic ideogram of the somatic complement. *N. poeticus*. Fig. 6. Somatic plate with fourteen chromosomes, a heteromorphic pair being satellited. Each of the B chromosomes shows a secondary constriction also. Fig. 7. Prophase nucleus showing attachment of the SAT-pair FD to the nucleolus. Fig. 8. Telophase showing two nucleoli. (Only one daughter-nucleus drawn.) Fig. 9. Diagrammatic ideogram of the somatic complement. (Only haploid complement represented.)

(4) One pair (GG) short with nearly median constrictions.

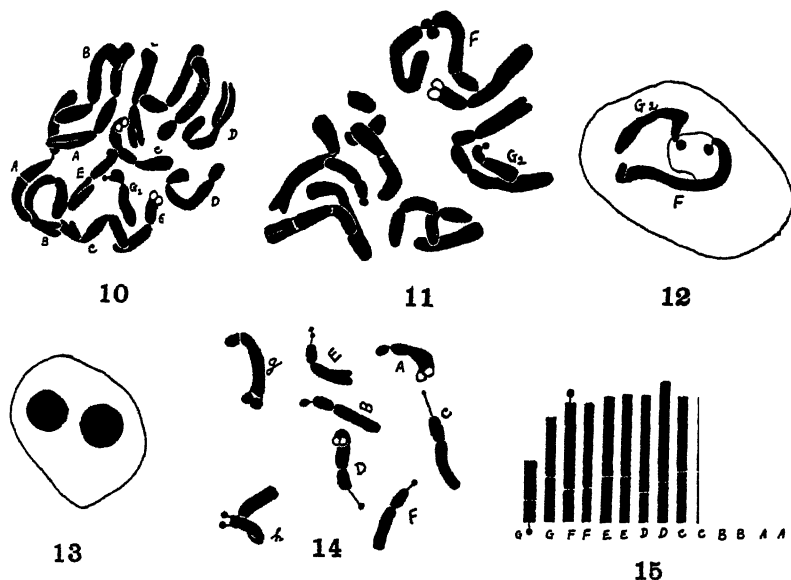
Fig. 7 represents a nucleus in prophase where the two SAT-chromosomes of unequal length are attached to the nucleolus. Whether the heteromorphic pair of satellited chromosomes in this species is the result of translocation or denotes hybridity, cannot be said with certainty.

In telophase two nucleoli corresponding to two SAT-chromosomes are organized (Fig. 8). The secondary constrictions of the B pair of chromosomes evidently do not take part in the organization of nucleoli.

(C) *N. gracilis* ( $2n = 14$ ).

The chromosome number in this species has been determined as  $2n = 14$  (Fig. 10). The somatic complement consists of (see ideogram Fig. 15):

(1) One longest pair (AA) with sub-medial primary constrictions and



FIGS. 10-15. (For magnification see description of Figs. 1-9.) *N. gracilis*. Fig. 10. Somatic plate with fourteen chromosomes. The A pair shows a secondary constriction on one of the arms. Fig. 11. Somatic plate with thirteen chromosomes and three fragments. A heteromorphic pair FG is satellited. Fig. 12. Prophase nucleus showing the heteromorphic SAT-pair  $FG_2$  attached to the nucleolus. Fig. 13. Late telophase showing two nucleoli. (Only one daughter nucleus drawn.) Fig. 14. SAT-chromosomes from different plates showing the variation in the size of the satellites. Figs. A-F represent one SAT-chromosome and  $g-h$  the other. Fig. 15. Diagrammatic ideogram of the somatic complement.

nearly median secondary constrictions on the longer arm. (2) one long pair (BB) with sub-medial constrictions.

(3) Four pairs (CC, DD, EE, FF) of medium length. Pairs CC and EE have sub-medial constrictions, whereas DD and FF are constricted sub-terminally. One of the F chromosomes bears a satellite on the distal end (Fig. 11).

(4) One pair (GG) small with sub-terminal constriction, one of them bearing a satellite on the proximal end (Fig. 11). The length of this pair was variable in some plates. In Fig. 10 one of them appears longer than the other; and also the spindle attachment region in this is sub-medial as compared to

sub-terminal in its supposed mate. This variation may be the result of translocation.

In Fig. 12 the heteromorphic pair of satellited chromosomes is seen attached to the nucleolus at prophase, and Fig. 13 shows two nucleoli in telophase corresponding to this pair of satellites. The secondary constrictions of the A pair of chromosomes do not take part in organization of nucleoli.

(D) *N. tazetta* var. *grand soleil d'or* ( $2n = 20$ ).

Nagao (1930) has reported the chromosome number of this variety as  $2n = 30$ , but my counts show that the number is  $2n = 20$  (Fig. 16). The somatic complement of this variety consists of (see ideogram, Fig. 23):

(1) One longest pair (AA) with nearly median primary constrictions. Each of these chromosomes has two secondary constrictions also, one on each arm.

(2) Three pairs long (BB, CC, DD) with sub-terminal constrictions. The D pair has, besides, a secondary constriction on the short arm.

(3) Two pairs (EE, FF) of medium length with sub-terminal constrictions; one of the F chromosomes bears a satellite on the proximal end (Fig. 22*b*).

(4) Four pairs (GG, HH, II, JJ) small with sub-terminal constrictions. The G pair is the biggest of the four and J the smallest. One of the I chromosomes bears a satellite on the short arm (Fig. 22*a*).

Fig. 19 shows the two satellited chromosomes at prophase, each attached to a separate nucleolus. In spite of the presence of a number of secondary constrictions only two nucleoli corresponding to the SAT-pair are organized in telophase (Fig. 21).

(E) *N. tazetta* (Mediterranean region) ( $2n = 21$ ).

The somatic number of this variety is determined to be 21 (Fig. 27). The somatic complement consists of:

(1) One pair longest (AA), with sub-terminal constriction. One of them bearing a satellite on the proximal end. (Fig. 28).

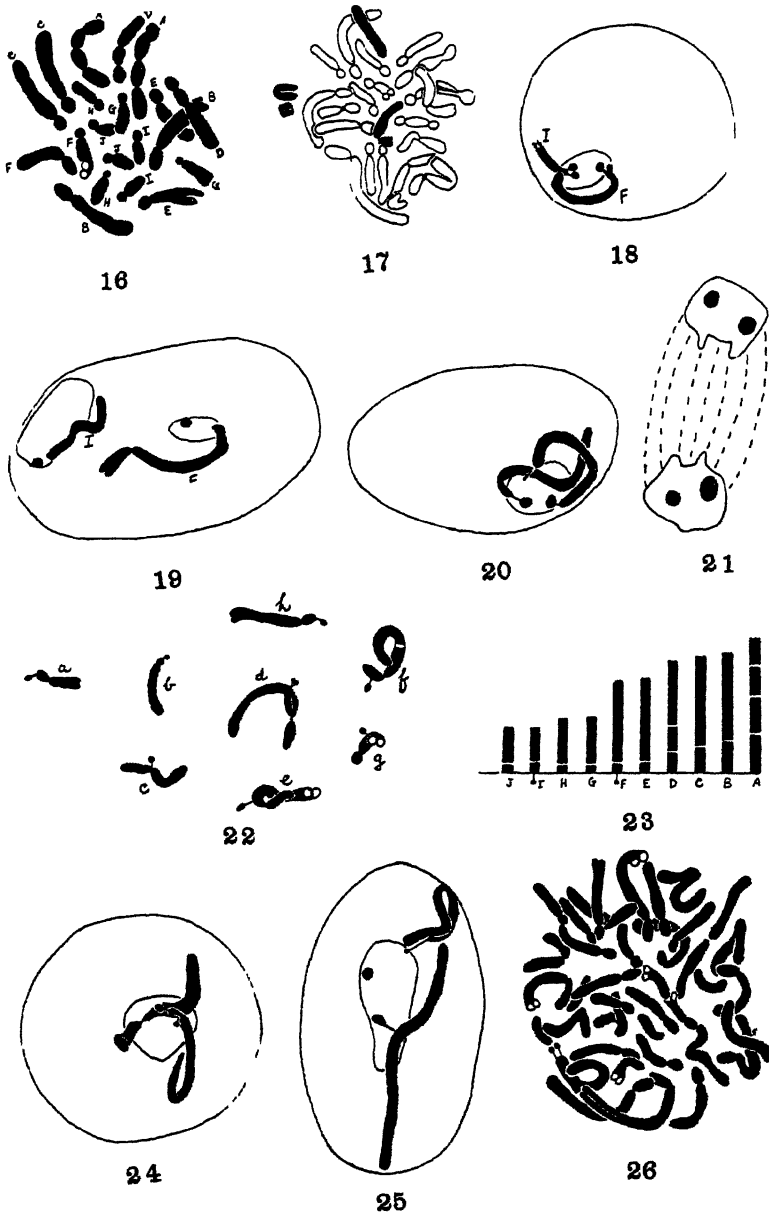
(2) Four pairs (BB, CC, DD, EE) long. The pair BB has a sub-median primary constriction, while all the others are constricted sub-terminally.

(3) Two pairs (FF, GG) of medium length. The pair FF is sub-terminally constricted, while GG has sub-median constrictions.

(4) Three pairs (HH, II, JJ) small with sub-terminal constrictions. Three of these bear satellites on the proximal end (Fig. 28). Besides the above there is an extra chromosome (marked K in Fig. 27) with almost terminal constriction. This is perhaps a fragment which has increased the chromosome number from 20 to 21.

Fig. 29 is a resting cell showing four nucleoli, two big and two small, corresponding to four satellited chromosomes. The significance of this will be discussed later.





FIGS. 16-26. (For magnification see description of Figs. 1-9.) *N. tazetta* var. *grand soleil d'or*. Fig. 16. Somatic plate with twenty chromosomes. Note the secondary constrictions of pairs AA and DD. Fig. 17. Somatic plate with nineteen chromosomes and five fragments. Fig. 18. Prophase nucleus showing the heteromorphic SAT-chromosomes FI attached to the nucleolus. Fig. 19. Prophase nucleus showing the larger SAT-chromosome F attached to the smaller nucleolus and smaller SAT-chromosome I to the larger. Fig. 20. Prophase nucleus

(F) *N. tazetta*, var. *canaliculatus* ( $2n = 29$ ).

The somatic number of this is determined to be  $2n = 29$  (Fig. 30). The chromosome complement consists of:

- (1) One pair longest (AA) with sub-terminal constriction.
- (2) Two pairs long (BB, CC) with sub-terminal constrictions.
- (3) Three chromosomes ( $D_1 D_2 D_3$ ) long with median constriction.
- (4) Two chromosomes ( $E_1 E_2$ ) long with sub-median constriction.
- (5) Four chromosomes ( $F_1 F_2 F_3 F_4$ ) long with sub-terminal constriction. These chromosomes are shorter in length than the BB and CC pairs.
- (6) Four medium-sized chromosomes ( $G_1 G_2 G_3 G_4$ ) with sub-terminal constrictions. All these bear satellites on the proximal end.
- (7) One pair (HH) of medium length having sub-median primary constrictions.
- (8) Four pairs (II, JJ, KK, LL) small with sub-terminal constrictions. One I chromosome has a secondary constriction also on the longer arm (Fig. 31).

In telophase six nucleoli are organized (Fig. 32). In this variety, evidently, the secondary constrictions also take part in the organization of the nucleolus besides the SAT-chromosomes. The significance of six satellites and six nucleoli in this variety will be discussed later.

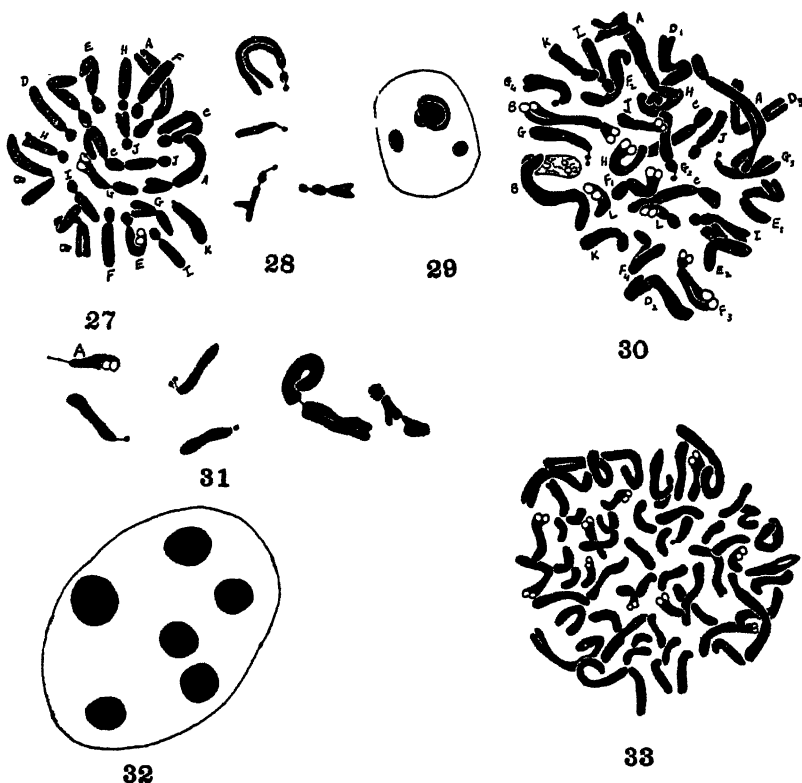
(G) *N. pseudonarcissus* var. *Victoria* ( $2n = 22$ ).

Nagao (1929) reports the somatic chromosome number of this variety as 14, but according to my observations it is 22 (Fig. 34). The somatic complement can be classified as follows:

- (1) Two longest chromosomes with sub-terminal constrictions.
- (2) Ten long chromosomes with sub-median constrictions. Two of these bear satellites on the long arm (Fig. 34) and two have median secondary constrictions on the shorter arm (Fig. 35). Besides these ten chromosomes there is another long chromosome (marked A in Fig. 34) with median constriction. It seems to have no mate in the whole complement.
- (3) Six medium-sized chromosomes with median or sub-median constrictions.
- (4) Four small chromosomes with sub-terminal or sub-median constrictions. One of these bears a satellite on the proximal arm.

showing one pair of SAT-chromosomes attached to the nucleolus. One of them is in contact with the nucleolus at both ends. Fig. 21. Telophase showing two nucleoli in each daughter nucleus. Fig. 22. Showing the normal (*a, b*) as well as the lateral trabants (*c* to *f*). In *g* and *h* the satellite is translocated to the distal and proximal ends respectively of two non-homologous chromosomes. (*a, b* are from the same plate; all the others from different plates.) Fig. 23. Diagrammatic ideogram of the somatic haploid complement. Figs. 24 and 25. Prophase nuclei showing a normal and a lateral satellite attached to the nucleolus. Fig. 26. Tetraploid nucleus with forty chromosomes.

In Fig. 36 the three SAT-chromosomes are seen attached to the nucleolus, and in telophase three nucleoli corresponding to these satellited chromosomes are formed (Fig. 40). The secondary constrictions present in a pair of chromosomes are, therefore, non-nucleolar.



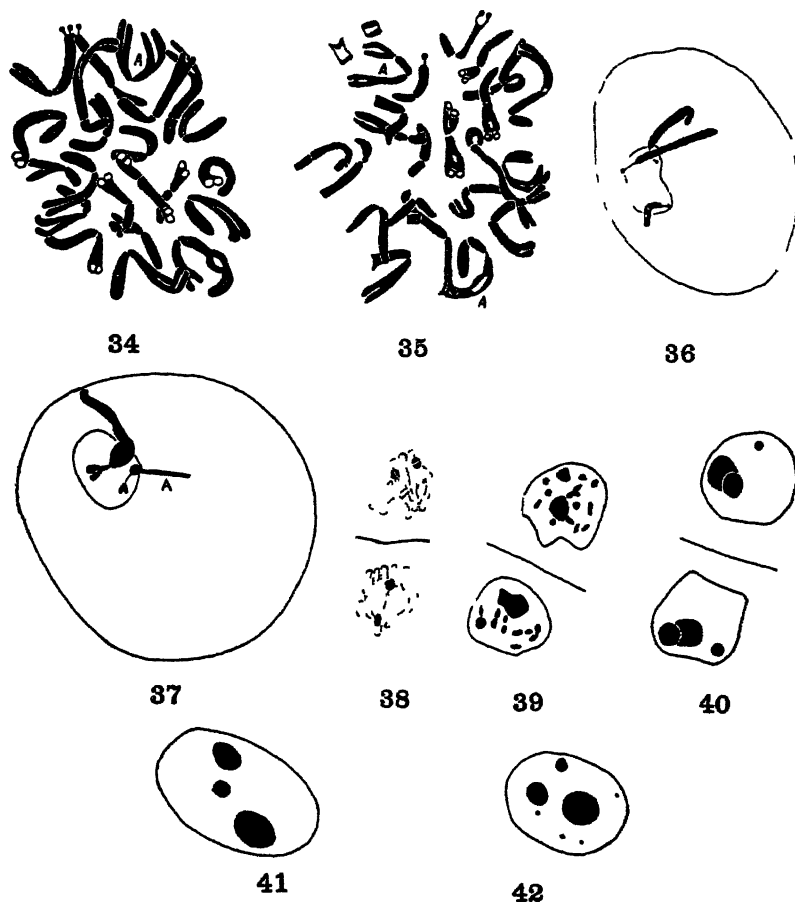
FIGS. 27-33. (For magnification see description of Figs. 1-9.) *N. tazetta* var. Mediterranean region. Fig. 27. Somatic plate with twenty-one chromosomes ('trisomic', K chromosome unpaired). Fig. 28. Four SAT-chromosomes drawn from the same plate. Fig. 29. Resting nucleus showing two big and two small nucleoli. *N. tazetta* var. *canaliculatus*. Fig. 30. Somatic plate showing twenty-nine chromosomes, four of which,  $G_1$ - $G_4$ , are satellited. Fig. 31. Showing four SAT-chromosomes and two secondarily constricted chromosomes. (The latter have been drawn from a different plate.) Fig. 32. Resting cell showing six nucleoli, one very big and the others smaller. Fig. 33. Tetraploid nucleus with fifty-eight chromosomes.

The morphology of the chromosomes on the whole shows that it is hyperallotriploid on the basic number of 7. The heteromorphic nature of the SAT-chromosomes also supports this view.

(H) *N. pseudonarcissus* var. *King Alfred* ( $2n = 28$ ).

The count of twenty-eight chromosomes in the root-tip cells of this variety (Fig. 43) agrees with that reported by de Mol (1922) and Nagao (1929).

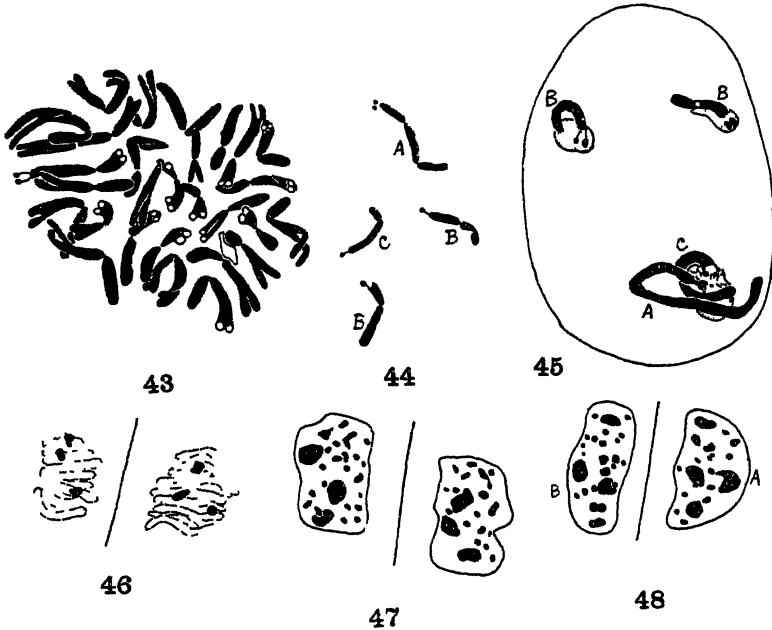
Nagao (1933), during the study of meiosis of this variety, observed a high



FIGS. 34-42. (For magnification see description to Figs. 1-9.) *N. pseudonarcissus* var. *Victoria*. Fig. 34. Somatic plate with twenty-two chromosomes, three of which are satellited. Fig. 35. Somatic plate with twenty-two chromosomes and three fragments. Note the secondary constrictions of the AA pair. Fig. 36. Prophase nucleus showing the attachment of three SAT-chromosomes to the nucleolus. Fig. 37. Prophase nucleus with two SAT-chromosomes attached to the nucleolus. The chromosome marked A shows a nucleolar body just below the satellite stalk. Fig. 38. Late anaphase showing the appearance of two small nucleoli in each daughter nucleus. Note the double nature of some of these nucleoli. Fig. 39. Early telophase showing one big and one small nucleolus in each daughter nucleus besides a large number of matrix droplets. (Chromosome threads not drawn.) Fig. 40. Late telophase showing three finished nucleoli of different sizes in each daughter nucleus. No matrix droplets are visible in any part of the nucleus. (Chromosome threads not drawn.) Fig. 41. Resting nucleus with three nucleoli. Fig. 42. Resting nucleus with three nucleoli and a few unfused matrix globules.

frequency of quadrivalent formation, from which he concluded that it is an autotetraploid. The analysis of somatic complement of this variety, however, does not support Nagao's conclusion as the chromosomes cannot be classified into seven sets of four chromosomes each. Moreover, the different

morphology of the four satellited chromosomes also shows that it must be an allotetraploid. One of the satellited chromosomes is very long, bearing a satellite on the long arm. The other three SAT-chromosomes are much smaller. Two of these bear satellites on the long arm and the third one on



FIGS. 43-8. (For magnification see description of Figs. 1-9.) *N. pseudonarcissus* var. *King Alfred*. Fig. 43. Somatic plate with twenty-eight chromosomes and one fragment. Fig. 44. Showing the four SAT-chromosomes in the somatic complement (drawn from another plate). Fig. 45. Prophase nucleus with three unfused nucleoli to which the four SAT-chromosomes are seen attached. Fig. 46. Late anaphase showing appearance of three small nucleoli in each daughter nucleus. Fig. 47. Early telophase with three prominent nucleoli in each daughter nucleus besides a very large number of matrix droplets of irregular shape. (Chromosome threads not drawn.) Fig. 48. Mid-telophase showing four prominent nucleoli in the pole A and three in the B, besides numerous matrix droplets. (Chromosome threads not drawn.)

the short arm (Fig. 44). These observations are confirmed from attachment of these chromosomes to the nucleoli at prophase (Fig. 45).

The history of this variety was kindly supplied by the Secretary of the Royal Horticultural Society, London. This shows that it originated from a cross between *N. pseudonarcissus* var. *Emperor* and *N. hispanicus* var. *maximus* (*N. major*). The former is a triploid variety with  $2n = 21$  chromosomes and the latter a diploid with  $2n = 14$ . Evidently the fertilization of an unreduced megaspore of the triploid variety by a reduced gamete from the diploid parent must have produced the present tetraploid. The morphology of SAT-chromosomes, described above, is in perfect harmony with this situation.

(I) *N. incomparabilis* var. *Sir Watkin* ( $2n = 21$ ).

The count of twenty-one chromosomes in this variety (Fig. 49) agrees with that reported by Nagao (1929).

The chromosomes of this variety are difficult to homologize, which is perhaps due to its hybrid nature. Many taxonomists regard this species as of hybrid origin, from *N. pseudonarcissus* and *N. poeticus*. The heteromorphic nature of the SAT-chromosomes also supports this view (Fig. 50). Fernandes has demonstrated a similar asymmetry of the chromosomes in a diploid variety of this species.

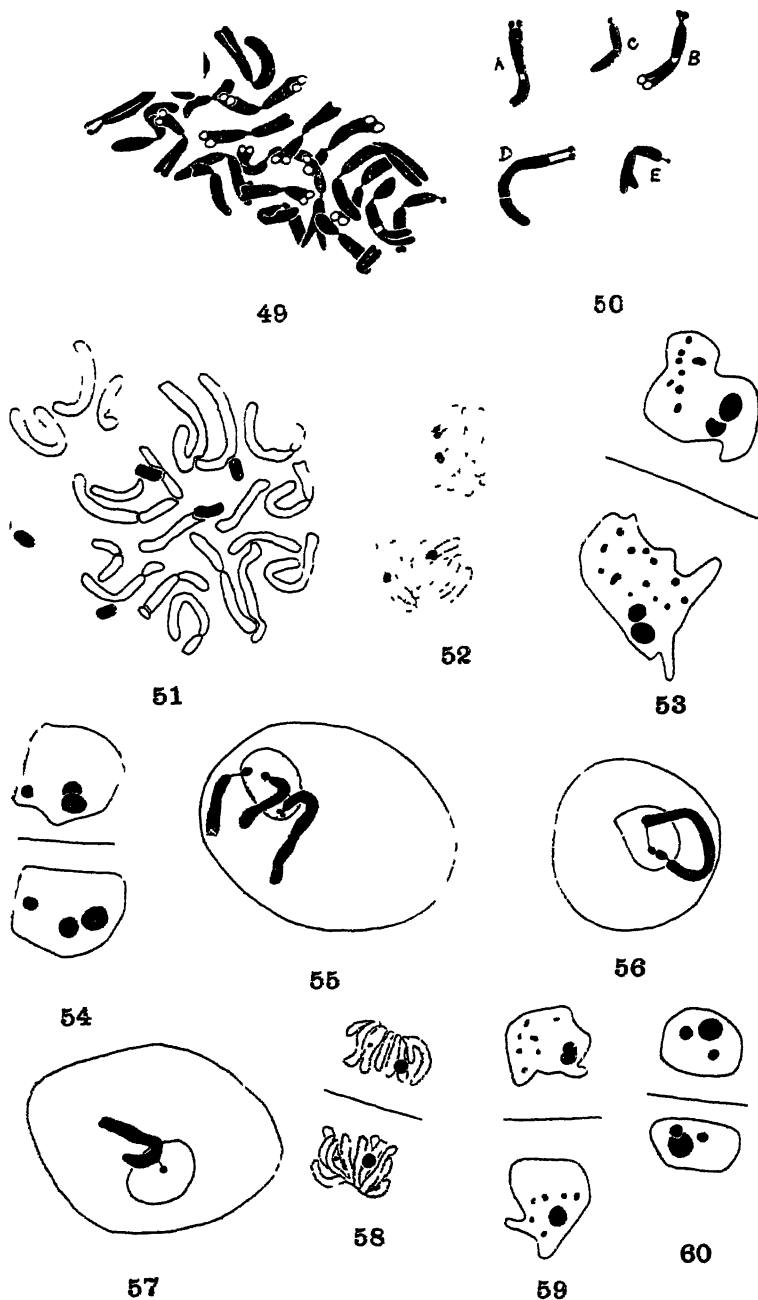
The presence of three satellites and three nucleoli (Fig. 54) in this variety denotes that it is a triploid on the basic number seven.

(b) *Cardinal Numbers in Narcissus*

The results of Stomps, de Mol, Nagao, Fernandes, and Sato in conjunction with those reported in this paper show that the chromosome numbers in *N. pseudonarcissus*, *N. incomparabilis*, *N. poeticus*, *N. odoratus*, *N. Jonquilla*, *N. bulbocodium*, *N. minor*, *N. reflexus*, and *N. Barrii* are multiples of seven. Fernandes (1931a, b) reports a basic number of six in four species (*N. rupicola*, *N. scarberulus*, *N. calcicola*, and *N. gaditanus*) and five in *N. tazetta*. Nagao (1933), however, disputes the existence of the basic number five in *Narcissus*, and thinks that *N. tazetta* has a basic number of ten.

The evidence presented in this paper on the number of satellites and nucleoli in three varieties of *N. tazetta* indicates that the cardinal number in this species is five. It has been shown earlier that a variety of *N. tazetta* from Mediterranean region with  $2n = 21$  chromosomes has four satellites and four nucleoli, which strongly suggests that it is a hypertetraploid on the basis of five and not a hyperdiploid on the basis of ten. Again, variety *canaliculatus* ( $2n = 29$ ) shows four satellites and two secondary constrictions corresponding to six nucleoli in telophase. On this basis it is a hypo-allohexaploid on the basis of five. The third variety, *grand soleil d'or* ( $2n = 20$ ), although a tetraploid, shows only a pair of satellites and nucleoli. The loss of the extra pair of satellites in this variety can be inferred through mutation or amphiplasty. This conclusion seems justified also on the grounds that the existing pair of satellites in this variety is heteromorphic.

The observations of Sato (1938) on the number of satellites and nucleoli in two different varieties of *N. tazetta* can also be adduced in favour of the reality of five as a basic number, although he could not understand the true significance of the situation. He finds in variety *papyraceus* ( $2n = 22$ ) four satellites and four nucleoli, which is quite consistent with its tetraploid nature. Again in the variety *suisen* ( $2n = 32$ ) he finds four SAT-chromosomes and four nucleoli. This shows that this variety is a hyperhexaploid in which two SAT-chromosomes have most probably been lost.



FIGS. 49-60. (For magnification see description of Figs. 1-9.) *N. incomparabilis* var. *Sir Watkins*. Fig. 49. Somatic plate with twenty-one chromosomes ( $3n$ ). Fig. 50. A, B, and C represent the normal SAT-chromosomes in the complement (drawn from another plate). D

Nagao (1933) has described the meiosis of the sacred Chinese Lily ( $2n = 30$ ), a variety belonging to *N. tazetta*. At metaphase I he found seven gemini in about 5 per cent. nuclei, eight in 37 per cent., and nine in 20 per cent. nuclei. If this variety were a triploid on the basis of ten, then multivalents higher than trivalents cannot be ordinarily expected at heterotypic metaphase. On the other hand, if it is taken to be a hexaploid on the basis of five, the higher multivalents so frequently observed by Nagao are quite easy to explain.

All the instances cited above strongly suggest that *N. tazetta* is based on the cardinal number five as originally reported by Fernandes. Therefore, the three cardinal numbers in *Narcissus* are five, six, and seven, and not six, seven, and ten. Further studies on this species should prove this point more conclusively.

Assuming five, six, and seven to be the cardinal numbers in *Narcissus*, it remains to be seen which of them is the primary basic number from which the other two have been derived. A comparison of the ideograms of the varieties of *N. tazetta* with those of other species having seven as the basic number shows that in the former the chromosomes, on the whole, are short in length and also subterminally constricted, while in the latter they are much longer and have mostly sub-median constrictions. On this ground, then, five cannot represent the primary basic number. On the other hand, a large majority of *Narcissus* species are based on the cardinal number seven, four on six, and only one has chromosomes in multiples of five. This also shows that seven may be the primitive basic number, from which five and six have been derived. This could happen either by fusion or loss of certain chromosomes. If fusion of one or more chromosomes had occurred, then in the somatic complement of species based on cardinal numbers five and six we expect two and one very long chromosome, respectively, which should also have sub-median constriction. As no material of the twelve-chromosomal species was available, this situation could not be verified in those, but none of the varieties of *N. tazetta* examined during the present course of study have shown this condition. Fusion of chromosomes, therefore, does not seem to be an adequate explanation. The only alternative hypothesis is that numbers five and six

and 8 show the variation in the size of the satellite of the A and C chromosomes respectively. Fig. 51. Somatic plate with twenty chromosomes and six fragments. Fig. 52. Early telophase showing the appearance of two small nucleoli of equal size in each daughter nucleus. Note the double nature of one of the nucleoli. Fig. 53. Slightly later telophase showing one big and one small nucleolus in each daughter nucleus besides many matrix droplets. (Chromosome threads not drawn.) Fig. 54. Late telophase showing three finished nucleoli in each daughter nucleus. No matrix droplets are visible at this stage. *N. pseudonarcissus* var. *Emperor*. Fig. 55. Prophase nucleus showing three SAT-chromosomes attached to the nucleolus. Fig. 56. Prophase nucleus showing a chromosome attached to the nucleolus at both ends by satellites. Fig. 57. Prophase nucleus showing a lateral trivalent attached to the nucleolus. Fig. 58. Late anaphase showing the appearance of one nucleolus in each pole. Fig. 59. Early telophase showing one big nucleolus in each daughter nucleus besides numerous matrix globules. Fig. 60. Late telophase showing three finished nucleoli in each daughter nucleus. The matrix globules have now completely fused with these.



have been derived from seven by a gradual process of reduction, as has taken place in more advanced species of *Crepis* (Hollingshead and Babcock, 1930), Gramineae (Avdulov, 1931), Polygonaceae (Jaretsky, 1928), and Cruciferae (Manton, 1932).

Delaunay (1926) was the first to postulate in *Muscari* that reduction in chromosome size takes place with advancing evolution. Babcock and Cameron (1934) noticed the same phenomenon in *Crepis*. More recently Babcock Stebbins, and Jenkins (1937) working with *Crepidinae* find that a great majority of the more primitive sub-genera as well as species have, on the average, definitely larger chromosomes than the more specialized ones.

Avdulov (1931), on the other hand, postulates an increase in chromosome size with advancing evolution. He connects this phenomenon with a progressive cooling of the climate during the evolution of these species with longer chromosomes. Avdulov's hypothesis, however, does not seem applicable to *Narcissus*, as most of the species belonging to this genus are native only to the Mediterranean region.

Levitsky (1931) and Levan (1935) suggest that primitive types tend to have chromosome sets of which the members are all about the same size, and which all possess median or sub-median constrictions, and that with advancing evolution the chromosomes become more unequal in size and tend to become subterminally constricted. This hypothesis is also borne out by Babcock *et al.* (1937) in *Crepidinae*.

On the postulates of Delaunay and Levitsky, therefore, *N. tazetta* (which has the cardinal number five) should be regarded as more advanced. This is also shown by the morphological characters of the plants. All varieties of *N. tazetta* have smaller flowers than the species based on the cardinal number seven. It therefore appears that, as in *Oenothera* (Gates, 1933), the small-flowered species are derived from those with large flowers.

Levan (1935) thinks that a change from median to sub-terminal constrictions is the result of unequal translocation. Working with two species of *Allium* whose chromosomes were nearly equal in size and had mostly median constrictions, he found that the majority of the translocations obtained resulted in chromosomes of unequal size and with more sub-terminal constrictions. Levan's hypothesis, though quite reasonable, is not borne out by observations of Navashin and Gerassimova (1935), who find that small chromosomes and short arms of chromosomes act most often as recipients of translocations produced in *Crepis*, and hence a progressively greater inequality in the size of chromosomes and chromosome arms may not be attained by this method. Levitsky (1931) thinks that the inequality in chromosomes is evolved by progressive accumulations of deficiencies. There is, however, no evidence so far to support his view.

### (c) *Fragmentation of Chromosomes*

Fragmentation of chromosomes was frequently observed in most of the varieties that were under study during the present course of investigation.

Fig. 2 is from *N. odorus*, showing twelve chromosomes and five fragments; Fig. 17 represents nineteen chromosomes and five fragments in *N. tazetta* var. *grand soleil d'or*; Fig. 11, thirteen chromosomes and three fragments in *N. gracilis*, Fig. 35, twenty-two chromosomes and three fragments in *N. pseudonarcissus* var. *Victoria*, and Fig. 43 shows twenty-eight chromosomes and one fragment in *N. pseudonarcissus* var. *King Alfred*. Most of these fragments appear to be devoid of spindle attachment regions.

In the behaviour of fragments much depends upon the spindle attachment region. Proximal fragments possessing a kinetochore behave in every respect as a normal chromosome irrespective of their length. On the other hand, in the case of distal fragments either of the following two things may happen:

(i) It may get fused with another chromosome (homologous or non-homologous) and persist as a translocation. In such a case the balance of the nucleus would not be affected, unless a 'position effect', such as has been genetically shown in certain translocations in *Drosophila* (Muller and Altenburg, 1930) comes into action. Among the simple translocations involving only homologous chromosomes, the first to be worked out both cytologically and genetically was that in which a portion of the Y-chromosome was found attached to the X-chromosome in *Drosophila* (Stern, 1926). Since that time a number of other examples involving various pairs have been discovered. Among translocations involving non-homologous chromosomes, the first known case is one in which a portion of X-chromosome was transferred to chromosome III in *Drosophila*. Another example of the same kind is that in which the right end of chromosome III was transferred to chromosome II (Painter and Muller, 1929). By crossing flies having this translocation with normal ones, hyperdiploid flies were obtained with a normal complement plus the translocated piece. Studies on the linkage relations in these flies showed between what genes the original break had occurred and also that the hyperdiploid flies possessed an excess of genes belonging to the right end of chromosome III. Many such translocations are known in plants also, such as *Zea*, although in the latter case the supposedly simple translocations have been proved to be reciprocal translocations (Burnham, 1932).

(ii) It may get lost, owing to its not fusing with a proximal fragment or any other normal chromosomes. Such a deletion might affect the viability of the cell or the organism, depending upon the amount and nature of the material eliminated. When the viability is not too seriously impaired, it may result only in genetic deficiency. The first known case of such deficiency was one in which a dominant gene for waltzing character in certain mice was lost along with a portion of a chromosome (Painter, 1927). In *Zea* the loss of genes *Pl* and *lg* responsible for purple colour of plant and liguleless characters respectively has been inferred in the same way.

The evolutionary value of fragmentation in establishing species or races with higher chromosome numbers is also an important point to consider. In many plant genera the increase in chromosome number has been inferred to

be due to transverse-segmentation of one or more chromosomes. In *Secale* (Gotho, 1924; Belling, 1925; Emme, 1928, and Levitsky, 1929) besides the fourteen chromosome races there occur others with sixteen chromosomes. The supernumerary chromosomes with sub-terminal constrictions in the latter group are supposed to have arisen by fragmentation of some long chromosomes with more or less median constrictions present in the 14-chromosome races. Metz (1916) describes a series of *Drosophila* species in which a higher chromosome number is thought to be due to the breaking up of long V-shaped chromosomes. Newton (1926) thinks that the chromosome number  $n = 16$  in *Tulipa galatica* has arisen by fragmentation of four long chromosomes found in the 12-chromosomal species of *Tulipa*. In *Vicia cracca* the 7-chromosomal races are supposed to have arisen by segmentation of a long chromosome present in 6-chromosome races (Sveshnikova, 1928). Heilborn (1924) has shown in *Carex*, that medium and small chromosomes have arisen by fragmentation of long chromosomes. Gates (1915) is of opinion that small chromosomes in some Liliaceous plants have arisen by transverse segmentation. Levan (1932) thinks that 18-chromosomal species of *Allium* have arisen from 16-chromosomal species by fragmentation.

Although fragmentation has so far been considered to be an important factor in chromosome evolution, more recent results of irradiation experiments have exposed the apparent difficulties in accepting fragmentation as a mechanism for bringing about increase in chromosome number. It is now a well known fact that a chromosome can function both in mitosis and meiosis only when it possesses the spindle attachment constriction containing the kinetochore. The observations on variations experimentally produced by X-rays (Navashin, 1931, in *Crepis*; Levitsky and Araratian, 1932, in *Secale* and *Vicia*, Dobzhansky, 1931, in *Drosophila*) or spontaneously arising in somatic mitosis have shown that fragments lacking kinetochores do not survive unless they join another chromosome or fragment possessing such a body. Since each chromosome has only one kinetochore, which has never been broken by X-rays, simple fragmentation cannot produce more chromosomes than there are kinetic bodies; in other words, fragmentation is unable to increase the chromosome number unless new kinetochores are developed *de novo* or this body itself fragments, about which no clear evidence exists at present. The first well-known case of presumed *de novo* formation of kinetic constriction in *Crepis* (Navashin, 1926) has later (1932) been considered to be uncertain by the same author. The second instance, reported by Darlington (1929), is still less conclusive. Thus in view of the constancy of the number of kinetic bodies in a chromosome complement, fragmentation (as well as fusion) cannot be regarded as so simple an evolutionary process as it has been considered hitherto. The only clear case of fusion of chromosomes is that inferred by Gates (1924) in *Drosophila* species, where two terminally constricted chromosomes have fused to give a V-shaped chromosome.

On the basis of this knowledge it follows that the only method of changing

the basic chromosome number is addition or elimination of one or more kinetic bodies through some process which does not affect the stability of the organism. Navashin (1932) has suggested the 'dislocation' hypothesis to explain the formation of new stable chromosome numbers. Although the appearance of *Drosophila* flies with three pairs of chromosomes in X-ray experiments (Dubinin, 1936), lends considerable support to Navashin's dislocation hypothesis, the obvious difficulty in this appears to be that such a process in nature takes several generations to complete and it is therefore unlikely that it would go on in a sexually reproducing plant, owing to the tendency of the sexual process to eliminate chromosome abnormalities. In asexually reproducing plants, however, fragmentation and fusion of chromosomes along the lines suggested by Navashin are quite possible. In *Narcissus*, varieties with hypo- and hyperdiploid chromosome numbers are very frequent, and it is quite possible that such forms may have arisen by fusion and fragmentation also, besides hybridization.

#### (d) *Translocation of the Satellites*

Several cases of translocation of satellite were observed in *N. tazetta* var. *grand soleil d'or*. Fig. 22*a, b* represents the normal pair of SAT-chromosomes in this variety. Both have a sub-terminal primary constriction and the satellite is borne on the proximal end of each. The normal SAT-pair is heteromorphic, one chromosome being small and the other of medium length. Fig. 22*h* represents a very long chromosome to which one of the satellites from the normal pair has been translocated on the proximal end. Another case of translocation is seen in Fig. 22*g*, where a small chromosome has a satellite on the distal end. Besides these, several cases were met with where the satellites had been translocated laterally to a non-homologous chromosome. These lateral satellites were located either at the point of attachment constriction (Fig. 22*c*) or any other part of the chromosomes (Fig. 22*d* to *f*). The cells showing these lateral satellites did not form any definite sector of the root, but were found mingled with other cells. This means that the translocations had occurred independently in each cell. The behaviour of these lateral trabants at anaphase is not known, and whether they would persist as such cannot be said with certainty. Levan (1932) has, however, described a case of lateral trabants in *Allium allegheniense*, where he found that the trabant divided normally into two and each of the divided halves passed to opposite poles along with the chromatids of the chromosomes.

Figs. 24 and 25 represent two cells in prophase where the lateral satellites are seen attached to the nucleolus. The fact that the lateral satellite has taken part in the organization of the nucleolus raises the important question regarding the point at which the break might have occurred at the time when the satellite was severed from the body of the chromosome. It has been shown by McClintock (1934) in *Zea* and Gates and Pathak (1938) in *Crocus*, that the nucleolus is organized on each chromatid of the telophase chromosome by a

special body located at the proximal end of the SAT-chromosome just below the point where the satellite filament arises. As will be shown later, the same position holds good in *Narcissus*. This means that the satellites in the above mentioned cases must have been severed along with a small piece of chromosome from the proximal end, so that the nucleolar organizing body was also translocated intact. Translocations of satellites in this way would, therefore, not hinder the organization of the nucleolus and, provided the lateral trabants persist in anaphase, the normal processes of the cell can go on unobstructed. Two cases of translocation of the satellite were noticed in *N. pseudonarcissus* var. *Emperor* also. Fig. 56 represents a prophase nucleus where a chromosome is seen attached to the nucleolus at both ends by satellites. In Fig. 57 another chromosome is attached to the nucleolus by a lateral satellite.

Anderson (1934) has reported an interesting case of a chromosomal interchange in maize involving the attachment to the nucleolus. In normal maize, chromosome VI bears the nucleolus near the end of the short arm, the attachment forming an enlarged reticulate region in the earlier prophase stages. Beyond this reticulate region is a satellite composed of four chromomeres. Following X-ray treatment, an interchange took place between the reticulate region of chromosome VI and nearly one-third of the way out on the long arm of chromosome IX. A common reticulate region was formed by normal chromosome VI and the interchanged chromosome. The changed linkage data showed genetically that the thread contains genes. If the satellite stalk contains genes the translocation of the satellite might involve 'position effect', as in *Drosophila*.

Sporadic occurrence of translocations of satellite have been observed both in plants and animals. In *Scilla*, Sato (1936) finds a satellite translocated to the proximal end of another chromosome. Resende (1937*b*) has recently described in species of *Aloe* several cases of translocations involving a satellite. In animals translocations of satellites have been reported by Dearing (1934) in *Amblystoma*.

Lateral trabants have been seen by Mather and Stone (1933) in *Crocus*, Levan (1932) in *Allium*, Darlington (1929) in *Tradescantia*. Swezy (1935) has figured such trabants in seven species of *Crepis*, where they arose as a result of spontaneous structural changes. Camara (1939) obtained lateral trabants in *Aloe* and *Vicia* in material treated with X-rays.

#### (e) *Variation in Size of Satellites*

In *N. gracilis* great variations in the length of the satellite stalk and the size of the satellite were noticed from cell to cell in the same root-tip. Figs 14 A-F illustrate such variations in the same SAT-chromosome. In A there is hardly any connecting thread and the satellite is very thick, almost equal to the segment of the chromosome. In B there is a short connecting thread, at the same

time the satellite is fairly stout. In C the filament is very long and the satellite is hardly visible. In D also the stalk is fairly long and shows a minute satellite at the end. In E the satellite presents the tandem condition. In F the satellite as well as the connecting thread are both short.

The other SAT-chromosome in the same species also showed characteristic variations. In Fig. 14 G the satellites appear like thick knobs, with almost no connecting thread. In Fig. 14 H there are short connecting filaments and the satellites are much thinner than in Fig. 14 G.

Fig. 31 A shows a SAT-chromosome from *N. tazetta* var. *canaliculatus* in which the filament is very long and shows two insignificant granules, one at the extreme end and the other in the centre.

Fig. 50 a and d represent the same SAT-chromosome from two different cells of *N. incomparabilis* var. *Sir Watkins*. In (a) there is no connecting thread visible and the satellites are fairly thick. In (d) both the satellites and thread are very stout.

Variations in the size of the satellite have been reported by several workers. Taylor (1926) observed minor differences between satellites in a given cell as between the corresponding pairs of different roots, and emphasized the effect of the action of fixing fluids of various types upon the prominence of the various features. M. Navashin (1926), from the study of the trabants of *Crepis Dioscoridis*, expressed the idea that the first steps in the formation of species may be seen in the very small changes which distinguish themselves through the dimensions of the trabants of the D chromosome in *Crepis* species. According to S. Navashin (1927) the individuals of *Galtonia candicans* belong to two different types as regards their chromosomes—symmetrical plants having the trabants of the same size and asymmetrical plants, the trabants of which are of dissimilar size, one small and one big. He also obtained one bulb, the root of which showed an intermediate condition as regards the size of the satellites. Emme (1925) observed polymorphism with regard to presence or absence of one or both satellites of a particular pair of chromosomes in some species of *Hordeum*. According to Medwedewa (1930) there are three races of *Crepis Dioscoridis*, one with large satellites, one with small satellites and a third heterozygous, which gives offspring of the three types in the ratio of 1 : 2 : 1.

Fernandes (1935), studying the satellites of *N. reflexus* and *N. triandrus*, found that both these species are very polymorphic in satellite shape. He reports all degrees of transition from forms whose size is equal to nearly half a chromosome branch to simple filaments, and even the absence of filaments. On these grounds he denies the existence of symmetrical and asymmetrical races in *Narcissus*. Later (1936) he divides the satellites into two classes: (a) heterochromatin satellites—that is, those having all the characters of heterochromatin and which remain attached to the nucleolus during telophase, interphase, and prophase; (b) euchromatin satellites—those which have the characters of euchromatin, since they undergo transformations during

the telophases and prophase and are not visible at the surface of the nucleolus during interphase and the first stages of prophase.

Sato (1937), in a study of the single- and double-flowered races of *Galanthus nivalis*, finds a great size variation of the filament of the satellite, conditions with a long connecting strand to those with no connecting thread having been seen in different individuals and even in the same individual. Mensinkai (1939) observed a great variation in the size of the satellite in several species of *Allium*. While some of the instances cited above show that the size of the satellite (including the filament) is a variable character, Resende (1937) finds in *Aloinae* that it is more or less a fixed character. In fact, he has been able to classify the types of satellites found in different species of *Aloe* into ten classes on the basis of their size.

It therefore appears that while we have at one extreme the symmetrical races of *Crepis*, and several species of *Aloe* in which the size of the satellite is supposed to be a fixed character, at the other extreme are the cases of *Narcissus*, *Allium*, *Galanthus*, in which this structure varies from cell to cell in the same individual. The latter type of variation, though very important to consider, has received little attention from cytological workers, and has, consequently, remained unexplained. This is due to the fact that the very nature of the trabant, including the SAT-filament, has till recent years remained a mystery. Heitz (1931) does not state how the filament arises, although he describes the nucleolus developing round about this filament. Moreover, he thought that the SAT-filament was devoid of nucleic acid. It has now been shown conclusively by Fernandes and by Mensinkai that the filament does contain nucleic acid and is Feulgen positive. McClintock makes a bare mention that the SAT-filament arises secondarily due to growth of the nucleolus.

About the nature of the satellite and how it arises, very little is known. Mensinkai (1939) is perhaps the first to offer a reasonable conception of the satellite. He has shown in *Allium* that the SAT-filament is in continuation of the chromonema of the chromosome and has a spiral of an order lower than the minor spiral possessed by the chromosome. He further adduces evidence to show that the satellite is only a rolled end of the chromonema. On this conception of the satellite the variations described above are easily explicable when we consider that the whole process of mitosis is only a spiralization and despiralization cycle of the chromosomes. A very long filament with no visible trabant at the end can result by complete unravelling of the spirals of the satellite. Similarly a massive trabant with a short or no stalk can result by close spiralization of the SAT-filament. These unravelling and close spiralization phenomena are most probably controlled by pH changes in the cell. The time at which the nucleolus, with which the satellite, including its filament, remains in close association from telophase onwards, gets dissolved, would also affect the size of the filament. If the nucleolus persists right up to metaphase, the SAT-filament cannot spiralize along with the body of the

chromosome, due to obstruction resulting from its attachment to the nucleolus, and would consequently remain long and stretched. On the other hand, if the nucleolus is dissolved early in prophase before the protein molecules of the chromosomes reach the iso-electric point, the SAT-filament would get spirialized along with the body of the chromosome and hence become short. The false tandem condition could result from fusion of gyres of the filament spirals under the action of the fixatives.

#### (f) Somatic Doubling

Tetraploid cells were noticed in two varieties. Fig. 33 is a cell from *N. tazetta* var. *canaliculatus* showing fifty-eight chromosomes. In this variety three such cells were come across intermingled with other cells having the diploid number of chromosomes. Fig. 26 represents a tetraploid cell with forty chromosomes from *N. tazetta* var. *grand soleil d'or*. In this variety only one such cell was seen.

Somatic doubling in cells of the root is now a recognized phenomenon in many plants. Tetraploid cells may arise by the failure of cross-wall formation between the daughter nuclei, thus resulting in a binucleate cell. By later fusion or simultaneous division of the nuclei tetraploid numbers may arise. Another process which may lead to the same condition is the splitting of the chromosomes to double their number without division of the nucleus. When this occurs in a zygote or in a young embryo, a tetraploid individual would arise directly.

Stomps (1910) put forth the hypothesis of nuclear fusion to account for somatic doubling, but his conception has been rejected by de Litardière (1923) and Langlet (1927) on the grounds that it could not explain adequately the paired condition of the metaphase chromosomes of tetraploid cells. According to them, true binucleate cells arise as a result of nuclear division without concomitant cell division.

De Litardière (1923) explained the formation of tetraploid nuclei as a result of two successive cleavages of the chromosomes in prophase. According to him, the split between the daughter halves after the first cleavage widens until each becomes independent of the other, but they do not separate, remaining in prophase until the second cleavage occurs. According to de Litardière's contention, therefore, prophase in which the doubling occurred would be of very long duration.

Lorz (1937) in *Spinacia*, formulates a different hypothesis for the origin of tetraploid cells, which embodies the conception of two successive cleavages but stipulates that both do not necessarily occur in prophase. Normally the cycles of chromosomal division and nuclear division are perfectly co-ordinated, so that one chromosomal division occurs for every nuclear division. Occasionally, however, due to some causes such as extreme vacuolation of the cells, the rate of the nuclear division may be retarded or that of chromosomal division accelerated. The division of the chromosomes and the separation



of division products occurring at a faster rate than the division of the nucleus as a whole, ultimately results in two chronological cleavages occurring within one nuclear cycle. On this hypothesis, therefore, the frequency with which doubling of chromosomes occurs is dependent upon the rapidity of development of the environmental factors which distort the normal synchronization of nuclear and chromosomal behaviour.

Somatic doubling in hybrids has the quality of restoring fertility. Thus in a sterile hybrid a tetraploid shoot may arise by somatic doubling and this shoot may be highly fertile (*Primula kewensis*, Newton and Pellew, 1929). De Mol (1937) has described an interesting somatic mutation in the hybrid *N. pseudonarcissus* ( $2n = 14$ )  $\times$  *N. poeticus* ( $2n = 14$ ) where not only the number of chromosomes was doubled but also the number of nucleoli in telophase. This should be a method of distinguishing tetraploid tissue.

#### (g) Organization of the Nucleolus

The process of organization of the nucleolus was studied in two species and four varieties, including *N. pseudonarcissus* var. *Victoria* ( $2n = 22 = 3b+1$ ) and *N. incomparabilis* var. *Sir Watkins* ( $2n = 21 = 3b$ ). As the whole process of nucleolus organization is essentially the same in all these varieties, a full description of it will be given only in variety *Victoria*. By employing the Feulgen-light green technique (Semmens and Bhaduri, 1938), which gives a differential staining of the chromosomes (deep purple) and nucleolar material (green) the whole process could be studied with great clearness.

The variety *Victoria* has three satellited chromosomes, two of which are bigger than the third (Fig. 34). The organization of nucleoli in this variety, as well as in others, begins after the chromosomes tend to form a fairly compact group at each pole (tassement polaire) at the end of anaphase. As the chromosomes at this stage have already lost their identity due to shortening and contortion of the chromonemata the actual appearance of nucleoli on the satellitic region cannot directly be demonstrated, but judging from the later history of the process it can be inferred with very little doubt that these nucleoli arise on a fixed locus of each SAT-chromosome present in the complement.

At the earliest stage of nucleolar development two tiny nucleoli of almost equal size appear in a large number of cases in the variety *Victoria*. In some cases one or both of these nucleoli show a clear cleft in their centre (Fig. 38) which suggests that a separate nucleolus is being organized by each of the nucleolar-organizing elements of the two twin chromatids of the satellited chromosome, as shown by Gates and Pathak (1938) in *Crocus*, Dearing (1934) in the salamander *Amblystoma*, and McClintock (1934) in *Zea*. As the components of each of these nucleoli grow, they shortly fuse into one globular body.

As the telophase begins with the laying down of the nuclear membrane

the chromosomes begin to despiralize. At the same time, the matrix of each chromosome, which had hitherto formed an unappreciably thin film round the chromonemata, is sloughed off its surface and collects in the form of very tiny masses of irregular shape throughout the nucleus (Fig. 39). These stain green with the light green dye in the same way as finished nucleoli. McClintock (1934) thinks that a complete release of the matrix from the chromatin is necessary before the chromatin can function properly in metabolism. This view also finds support in Kuwada's (1926) experiments of artificial unravelling of the chromonemata in the pollen mother cells of *Tradescantia* by exposure to ammonia. Although the superficial appearance of the resting nucleus was brought about no nucleolus was formed. Thus there appears to be a close relationship between the release of matrix material on the one hand and organization of nucleolus and metabolic activity of the chromosomes on the other.

The matrix material, after its formation, begins to run together and at the same time these bodies seem to approach the two small nucleoli which had arisen at an earlier stage. One of these nucleoli now begins to grow at a faster rate than the other. In Fig. 39 it can be seen that one of the nucleoli has become much larger than the other, although in the beginning both were of the same size. This shows conclusively that the organizer of one SAT-chromosome is much more active in collecting nucleolar material than the other. In later stages, these two nucleoli keep growing in size, one faster than the other, and at the same time the matrix globules go on decreasing in number, till at the end of telophase we find only three finished nucleoli of different sizes, all the matrix material having merged into these (Fig. 40). The third nucleolus, evidently, appears at a very late stage and as most of the matrix material has been used up in building up the two nucleoli which arose first, its size is bound to be the smallest of all. In very few cases some of the tiny matrix globules do not get collected at the organizer and these, therefore, may persist during the resting stage (Fig. 42) or even up to prophase. However, judging from their extremely small size and also from the fact that they are not found attached to any chromosome at prophase, there can hardly be any doubt that they represent the remnants of the nucleolar material which did not get merged with the finished nucleoli.

An exactly similar process was noted in *N. incomparabilis* var. *Sir Watkins*. This variety being a triploid has three SAT-chromosomes in the somatic complement. Fig. 52 represents the earliest stage of nucleolar development, in which two small nucleoli have appeared earlier than the third. No other globules are yet visible in any part of the nucleus. In Fig. 53, which represents a slightly later stage, a number of matrix globules can be seen, besides the two original nucleoli which have grown in size, one of them more than the other. Fig. 54 is a very late telophase showing three finished nucleoli corresponding to three SAT-chromosomes in the somatic complement. Here all the matrix globules which were contributed by all the chromosomes during

the earlier stages have merged into the finished nucleoli. The third nucleolus, which appears last of all, is the smallest in size, evidently due to less matrix material being available for it.

The triploid variety Emperor ( $2n = 21$ ) affords equally significant evidence. As a sufficient number of flat metaphase plates were not available in this variety, the number of satellited chromosomes could not be determined at that stage, but judging from the attachment of three chromosomes to the nucleolus in prophase (Fig. 55) it can be inferred that it has three nucleolar chromosomes, as is expected in a triploid. This is further confirmed by the fact that in telophase a maximum of three nucleoli are organized.

The process of organization of the nucleoli in this variety is essentially the same as in the above mentioned two cases, with the difference that only one nucleolus originates in the earliest stage of nucleolar development (Fig. 58). At a later stage the matrix globules arise on the surface of chromosomes and this nucleolus grows in size (Fig. 59). The other two nucleoli do not appear until very late telophase when almost all the visible matrix globules have been merged into the nucleolus which arose first, the result being that both these nucleoli remain very small in size. Fig. 60 represents a late telophase where one large nucleolus and two very small ones can be seen in each daughter nucleus, no other matrix globules being visible in the nucleus at this stage.

Variety King Alfred is a tetraploid with four satellited chromosomes, one of which is bigger than the other three (Fig. 44). In very early telophase three small nucleoli were seen to arise in each daughter nucleus in a large number of cases (Fig. 46). In Fig. 47, which represents a slightly later stage, innumerable globules of matrix material are visible besides the three nucleoli which have now grown in size. In Fig. 48, which is very nearly mid-telophase, the three nucleoli have grown further in size and a fourth small one has appeared. It is more marked in the daughter nucleus marked A. In this figure plenty of matrix globules can still be seen. By the end of telophase all these get fused with the nucleoli and are not traceable in any part of the nucleus. However, at this stage mostly three finished nucleoli were seen. This reduction in number is most probably due to fusion of two nucleoli at an earlier stage.

The evidence from four varieties, presented above, points to the following conclusions:

(i) In *Narcissus*, nucleolar material is contributed from the matrix of chromosomes in the complement in the form of small globules, which get accumulated at definite loci of the SAT-chromosomes, most probably under the influence of their respective nucleolar bodies.

(ii) The metabolic activity of the nucleolar-organizers of various SAT-chromosomes varies. It is manifested not only in the accumulation of more or less matrix (nucleolar) material, but also brings the time factor into play, by which some nucleoli are organized earlier than others. Both these factors in turn control the ultimate size of the nucleolus.

The present observations agree with those of some of the earlier workers. Van Camp (1924) showed that nucleoli originate from chromosomes in telophase in the form of small globules which later by fusion form one large nucleolus. Derman (1933), in *Callisia* and *Paeonia*, and McClintock (1934), in *Zea*, have also recorded similar results. Derman, however, thinks that there are no definite nucleolus-producing chromosomes and that the association of nucleolus with the satellite is only a matter of chance. In view of the regularity with which SAT-chromosomes have been seen in association with the nucleolus in so many plant genera, Derman's conclusion of chance association becomes invalid.

#### 4. DISCUSSION

The exact role played by the nucleolus in the economy of the cell is still imperfectly understood. The greatest advance in the study of the nucleolus is the understanding of the mode of its organization in plant cells. Although several earlier investigators (Farmer, 1895; Wager, 1904) noted some connexion between the nucleolus and the network of the nucleus, Latter (1926) was the first to establish a definite morphological relationship between the nucleolus and the chromosomes in *Lathyrus odoratus*. Later, Sheffield (1927) and Gates and Sheffield (1929) established a similar relationship between the nucleolus and chromosomes in the P.M.C.'s of *Oenothera*.

Another line of observation which led towards the understanding of the mode of organization of the nucleolus began with the discovery of satellites on the chromosomes of *Galtonia* by S. Navashin (1912). Since then, although several workers observed connexion between the SAT-chromosomes and nucleolus (Sorokine, 1924, 1929; M. Navashin, 1925; Baranov, 1925; Senjaninova, 1926; Metz, 1927; Kuhn, 1928), the true significance of this relationship was not understood before the work of Heitz (1931), who pointed out that the nucleolus originates in telophase at the position of the satellite stalk or secondary constriction (which according to his conception were synonymous) and that the number of nucleoli formed in telophase nuclei corresponds to the number of SAT-chromosomes present in the complement.

As far as the relation of satellited chromosomes to the nucleolus is concerned, Heitz's hypothesis has received ample support from different plant genera, either directly, that is, from observations of actual attachment of SAT-chromosomes to the nucleoli, or indirectly, that is, from observations of numerical correspondence between the number of satellites and nucleoli in the somatic tissue. As examples may be quoted *Galtonia* (Smith, 1933), *Allium* (Levan, 1935, 1936; Mensinkai, 1939), *Crepis*, *Fritillaria*, and *Lilium* (Matsuura, 1935, 1937), *Scilla* and several genera of *Amaryllidaceae* (Sato, 1936, 1937, 1938), *Aloinae* (Resende, 1936, 1937), *Triticum* (Bhatia, 1938), and *Cicer* (Iyengar, 1939). In the present observations on *Narcissus* also such a relationship holds good.

But when we come to consider the relation of secondary constrictions to the

nucleolus some obvious difficulties arise. Kaufmann (1934, 1937) in *Drosophila*, Sato (1936, 1937) in *Gasteria*, *Haworthia*, *Scilla*, and *Galanthus*, Fernandes (1936) in some species of *Narcissus*, Sinoto (1937) in *Rumex*, Okuno (1937) in *Lobelia*, and Resende (1937) in some species of *Aloe*, find that some secondary constrictions do not take part in the organization of a nucleolus. In the present study also secondarily constricted chromosomes in addition to satellited ones have been seen in five different varieties (cf. Table I), but nucleoli are produced by such constrictions only in one of them, namely, *N. tazetta* var. *canaliculatus*. Some secondary constrictions are therefore nucleolar, while others are not. This necessitates a differentiation in these two kinds of secondary constrictions in order to explain the discrepancies that have lately arisen in several plant genera with regard to the numerical correspondence between such constrictions and nucleoli.

From a comparison of Fig. 16 and 31, which illustrate the secondary constrictions of *N. tazetta* varieties *grand soleil d'or* and *canaliculatus*, respectively, it is seen that the constrictions in the latter are much deeper and are characterized by a clear achromatic area. These are the nucleolar secondary constrictions. As opposed to these, the secondary constrictions of variety *grand soleil d'or* give the appearance as if they have arisen simply by twists of the chromonemata, due perhaps to action of the fixatives; or alternatively these constrictions arise at the points where there is a reversal in the direction of coiling of the chromonemata. The existence of an achromatic area in a nucleolar secondary constriction is to be expected on theoretical grounds also. The nucleolus being in close contact with this region from the time of its organization in telophase until the time of its disappearance in late prophase, it cannot undergo spiralization along with the body of the chromosome and hence remains despiralized. The chromonema at this region will be further stretched by the growth of the nucleolus and, therefore, appears filamentous, as is the case with the stalk of the satellite. Such secondary constrictions, therefore, must have a different appearance from the non-nucleolar constrictions and it should be possible to distinguish the two on this criterion. Whether these nucleolar secondary constrictions arise by the inversion or translocation of the 'region nucleogenique' of the satellite still remains to be proved. Matsuura's observations in *Trillium* and Paris suggest that the 'E' chromosome with satellite of Paris may be the result of translocation of the distal end of the 'A' chromosome of *Trillium*. Generally, these secondary constrictions have been considered lower in evolution than the satellite (Parthasarathy, 1939). Although this view might find support in Chen's (1936) observation on Protozoa, where he finds that the nucleolus surrounds a portion of the chromosome at a non-terminal region, at which a constriction might have been evolved later in evolution, there is no clear proof to show that satellites are a higher evolutionary type than secondary constrictions.

It was the belief of Heitz that the nucleolus was formed by the stalk of the satellite or secondary constriction. McClintock (1934) has shown, however,

in *Zea*, that the nucleolus originates not from the stalk of the satellite but from an organized body in the chromosome directly adjacent to the stalk of the satellite. According to her view, the stalk is very likely produced as the result of the growth of the nucleolus during telophase. Fernandes (1936), working with *Narcissus*, confirms McClintock's observations that the nucleolus is organized by a definite heterochromatic body, but thinks that the position of this body is not fixed. In *N. bulbocodium* var. *genuinus* he found one pair of chromosomes attached to the nucleolus at their terminal points. On the basis of this he introduced the term 'region nucleogenique' which, according to his view, may be terminal or sub-terminal. In the present study no cases of absolute terminal attachments of chromosomes to the nucleolus were observed. On the other hand, definite heterochromatic bodies similar to those observed by McClintock in *Zea* were seen in certain cases on the proximal end of the SAT-chromosome just below the point where the SAT-filament arose. Fig. 37 is a cell in prophase in *N. pseudonarcissus* var. *Victoria*, where such a body is clearly seen in chromosome marked 'A' at the point of union of this chromosome to the nucleolus.

Matsuura (1935, 1938) has reported some novel cases in *Trillium*, where he finds that one long (A) chromosome and also a short (E) chromosome have no satellites and are attached to the nucleolus terminally. He, therefore, denies the existence of any morphologically distinguishable body of heterochromatic nature which is responsible for the organization of the nucleolus. According to his view, all chromosomes of a complement possess functional activity for nucleolar development, but some of them become specialized due to differential 'valency', which brings about a competition amongst the chromosomes in their nucleolar-forming activities. The present author is inclined to agree with Matsuura in so far as each chromosome of a complement is considered capable of contributing nucleolar material towards the organization of the nucleolus, but before his view about the non-existence of satellites or nucleolus-organizing body can be accepted much more extensive study is needed. Resende (1937) has observed many satellites of extremely small size in some species of *Trillium*, where these satellites were previously supposed to be absent. Mensinkai (1939a) has demonstrated a minute satellite in *T. sessile*. Further studies on other species of *Trillium* may, perhaps reveal the presence of satellites in them also, or they may be in some cases below the limits of visibility.

Another line of study of the nucleolus has been in connexion with its chemical composition. The results of various investigators who have been engaged in this study indicate that chemically the nucleolus is not universally of the same composition. Zacharias (1885) reported that it contains proteins and plastin and no nucleic acid. Zirkle (1928) finds that the nucleolus does not contain nucleoproteins or nucleic acid, and he called the nucleolar material 'plastin'. Heitz (1931), using the Feulgen nuclear reaction, found no evidence for the presence of nucleic acid in the nucleolus. On trying

Feulgen reaction on the root-tip sections of *Narcissus*, I also find that nucleic acid is absent from the nucleolus, which was not visible without a counter-stain. Shinke and Shigenaga (1933), from their studies on the root-tips of *Vicia* and other plants, conclude that the material of the nucleolus is likely to be of lipoidal nature. This was also reported earlier by Fels (1926), who found in *Vicia faba* 'lipoid staining definitely positive', while Millon's reaction was comparatively indefinite. From all this evidence it appears that the nucleolus, though mainly containing lipoids, may be not totally devoid of nucleo-proteins, which vary in quantity at different stages in nuclear cycle and in different organisms.

The exact nature of the material which gives rise to the nucleolus in telophase is still a debatable point, but it appears that it originates in the matrix of the chromosomes. The work of Shinke and Shigenaga (1933) and others strongly suggests such a probability. They found that the matrix of the chromosomes is mainly composed of lipoids. This conclusion is based on the fact that the matrix is far more easily dissolved by lipoidal solvents than the spiral portions of the chromosomes. Further, they found that the matrix stains with lipid staining dyes.

The present study of *Narcissus* also affords evidence with regard to the identity of the matrix and the nucleolar substance. It has been shown that in telophase the matrix material of the chromosomes, after it is liberated from their surface, collects in the form of bodies of varying size, which take the same stain (green) as the nucleoli. It has also been shown that these matrix globules, after their formation, gradually get collected at fixed loci on the nucleolus-organizing chromosomes, thus giving rise to the finished nucleoli. Although the specificity of the light green dye as a test for lipoids does not follow from this, nevertheless the fact that the matrix and the nucleoli stain alike does suggest the identity of the two substances.

Accepting the argument that the nucleolus is composed mostly of lipoids, it remains to be seen how the latter substance arises from chromatin (nucleo-proteins and nucleic acid) of the chromosomes. The origin of lipoids from nucleoproteins has been demonstrated in the case of animal cells by Cannon (1932) and is said to depend on hydrolysis of the nucleoproteins which breaks them up into nuclein, and further hydrolysis breaks the latter into proteins and nucleic acid. The lipoids are said to originate from the proteins thus formed by a complex series of reactions. In plants also the lipoids are most probably formed by the same series of reactions, although direct evidence in this connexion is lacking. However, the microchemical tests of matrix performed by Shinke and Shigenaga (1933) do prove this fact indirectly.

Granting that the nucleolus is derived from the accumulated matrix material, its attraction to particular loci in the nucleus is another important problem requiring consideration. McClintock (1934) attributes it to the 'metabolic activity' of the organizer. The evidence for such an activity is that when the organizer is absent or is influenced by certain 'genomic

deficiencies' and thus ceases to function, the nucleolar substance is scattered in droplets instead of being organized into a finished nucleolus. Mensinkai (1939) explains this phenomenon on the basis of 'cataphoresis'. He suggests that the nucleolar globules consisting mainly of fats are electrically attracted to the organizer, which might behave as a pole with an opposite charge. Pure lipoids are, however, known to be non-electrolytes, but if a protein layer be supposed to envelop these fat globules then they can get charged electrically. Whether such a layer of protein exists around these globules is a matter for future research to decide. Again, to explain the accumulation of nucleolar material at the organizer requires the further assumption that the local positive charge on the nucleolar body is much greater in comparison with the charge on other parts of the chromosomes. The latter may perhaps be possible in the same way as the kinetochores of the chromosomes are supposed to be more strongly charged than the other parts during the anaphasic separation. The hypothesis of cataphoresis, though very attractive, seems to involve certain knotty problems which require to be solved by further research.

An analysis of the factors controlling the size of the nucleolus is another vexed problem. Heitz (1931a) noticed that when two SAT-chromosomes are present in a nucleus, distinctive size differences of the nucleoli can result. This was interpreted by him on the assumption that such differential activity of the two SAT-chromosomes is a function of the length of the satellite stalk. The work of McClintock, Sato, Fernandes, and Matsuura has, however, shown clearly that the stalk of the satellite is of secondary origin as a result of the growth of the nucleolus, and is, therefore, indispensable for its organization. Moreover, present observations, as well as those of Sato (1937) in *Galanthus*, Fernandes (1936) in *Narcissus*, and Mensinkai (1939) in *Allium*, have shown that the length of the stalk is a variable character, differing from cell to cell in the same individual. In the light of these findings, therefore, Heitz's hypothesis requires to be modified by postulating that the size of the developing nucleolus is responsible for the length of the satellite stalk and not the reverse.

McClintock (1934) has shown in *Zea* that where the two SAT-chromosomes are present in monoploid complement, the size of the nucleolus developed by each chromosome depends upon the relative functional capacity of the nucleolar organizing bodies. Basing her conclusions on the observations of size of nucleolus in the four types of spores produced by plants heterozygous for the interchange, she remarks

'If the nucleolar organizing elements have similar functional capacities, nucleoli of similar size will be formed. If, however, there is differential rate of functioning the nucleolar element with the greater rate of functional capacity will develop the larger nucleolus, that with smaller rate of functional capacity will develop a smaller nucleolus. When a nucleolar-organizing element with a slower rate of functional capacity is present alone, it develops just as large a nucleolus as a nucleolar-organizing element with a more rapid rate of functioning.'



The present observations are in perfect harmony with McClintock's findings. As shown earlier in this paper, *N. pseudonarcissus* var. *Victoria* is a hypertriploid having three satellited chromosomes in the somatic complement, and corresponding to these three nucleoli are organized in telophase. Two of these nucleoli arise earlier than the third, which can be attributed to the fact that two SAT-chromosomes are more active than the third in organizing their respective nucleoli. This point is further illustrated by the fact that although the nucleoli just after their formation in early telophase are almost of equal size, one of them in later stages goes on gradually increasing in size. The organizing body of this particular SAT-chromosome which organizes the largest nucleolus has, therefore, the capacity of collecting more matrix (nucleolar) material than the other. The length of the SAT-chromosome or the size of the satellite cannot be taken to have any relation with the size of the nucleolus, as no marked difference is visible in these two satellited chromosomes with regard to these characters. Exactly similar observations are recorded in *N. incomparabilis* var. *Sir Watkins*. That the length of the SAT-chromosome has no relation with the size of the nucleolus can be shown also by another example. In *N. tazetta* var. *grand soleil d'or* there is a heteromorphic pair of satellited chromosomes. In Fig. 19, which represents a nucleus in prophase in this variety, the longer SAT-chromosome is attached to the smaller nucleolus and the smaller one to the larger nucleolus.

This evidence on the differential activity of the nucleolar bodies can help to explain the peculiar observations of 'differential amphiplasty' reported by Navashin (1934) in species hybrids of *Crepis*. In these hybrid plants if the rate of functioning of the nucleolar organizing element of the SAT-chromosome of one species is far greater than that of the other species, competition between them might totally inhibit the activity of the weaker organizer and no nucleolus would consequently be formed by that chromosome. In the absence of a nucleolus the satellite stalk also will not appear. Thus the disappearance of the satellite in *Crepis* hybrids implies that the satellite has fused with the body of the chromosome owing to the failure of development of the nucleolus at its particular region. The truth of this is further shown by the fact that the amphiplastic effect vanishes and the SAT-chromosome begins to function normally when the hybrid is back-crossed to one of the parental species.

Lesley (1938) describes three races differing in size of the 'A' or nucleolar chromosomes in *Solanum Lycopersicum*. The difference between the three types of 'A' chromosomes (short 'A', long 'A' and very long 'A') is presumed to be due to addition of satellite material. The volume of satellite is approximately doubled in long 'A' as compared with short 'A' and in the very long 'A' as compared with long 'A'. The nucleolus averages larger in races with two long 'A' chromosomes than in races with two short 'A's and still larger in the plant with two very long 'A's. She also finds that the very long 'A' chromosome is attached to the nucleolus at two points, whereas the short 'A'

and long 'A' are attached at only one point. This is a new situation which requires to be elucidated by further studies.

## 5. SUMMARY

1. Somatic chromosomes of nine varieties belonging to six species of *Narcissus* were studied with special reference to number of satellites and nucleoli.

(a) *N. odoratus* ( $2n = 14$ ), *N. gracilis* ( $2n = 14$ ), and *N. poeticus* ( $2n = 14$ ) are diploids, each with a pair of satellites and two nucleoli. *N. pseudonarcissus* var. *Victoria* ( $2n = 22$ ) is a hypertriploid with three satellites and three nucleoli. *N. pseudonarcissus* var. *King Alfred* ( $2n = 28$ ) has four satellites and four nucleoli and is a tetraploid. *N. incomparabilis* var. *Sir Watkins* ( $2n = 21$ ) is a triploid with three satellites and three nucleoli. The chromosome numbers in these five species are based on the cardinal number seven.

(b) Evidence from the number of satellites and nucleoli in some varieties of *N. tazetta* suggests that five is the basic number in this species. A variety with  $2n = 21$  chromosomes from the Mediterranean region showed four satellites and four nucleoli and is, therefore, a hypertetraploid. Var. *canaliculatus* ( $2n = 29$ ) shows six nucleoli corresponding to four satellites and two nucleolar secondary constrictions and is a hypohexaploid. Var. *grand soleil d'or* ( $2n = 20$ ) has only a heteromorphic pair of satellited chromosomes and two nucleoli. The loss of the second pair of satellites by mutation or amphiplasty is, therefore, indicated. Some further evidence for the reality of five as the cardinal number in *N. tazetta* has also been adduced.

(c) Judging from the morphology of chromosomes, species with seven as the cardinal number appear to be the most primitive. The derivation of cardinal numbers five and six from seven has been discussed.

(d) The secondary constrictions of *N. poeticus*, *N. gracilis*, and *N. tazetta* var. *grand soleil d'or* do not take part in the organization of nucleoli.

2. Study of the process of organization of nucleoli in one hyper-triploid, two triploids and one tetraploid variety has led to the following conclusions:—

(a) The nucleolar material is supplied by all the chromosomes in the complement in the form of small globules derived from the matrix, which get accumulated at definite loci of the SAT-chromosomes, probably under the influence of their respective nucleolar bodies.

(b) The functional activity of the nucleolar-organizing elements of the various SAT-chromosomes varies. It is manifested not only in the accumulation of more or less nucleolar material, but also brings the time factor into play, by which some nucleoli are organized earlier than others. Both these factors in turn control the ultimate size of the nucleoli.

3. Some interesting features, e.g. fragmentation of chromosomes, translocation of satellites, variations in the size of the satellite including its filament, somatic doubling, &c., are described.

4. The relation of SAT-chromosomes and secondary constrictions to the nucleoli, composition of the nucleoli and the factors controlling their size are discussed.

## 6. ACKNOWLEDGEMENTS

In conclusion I must express my sincerest thanks to Professor R. Ruggles Gates, for sympathetic guidance, valuable criticism and adequate facilities for these investigations.

I am also deeply indebted to Mr. H. R. Stewart, Director of Agriculture, Punjab, for relieving me of my official duties in order that I might undertake this study.

My thanks are also due to the Royal Horticultural Society, London, for supplying me with data of the history of some varieties of *Narcissus*.

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# Investigations on the Significance of Ethereal Oils in Regulating Leaf Temperatures and Transpiration Rates

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With twelve Figures in the Text

## INTRODUCTION

IN the middle of the last century the physicist Tyndall (1862) made the discovery that the vapours of certain essential plant oils showed a very great absorptive power for radiant heat. The teleological application of this fact seems to have attracted botanists, for Haberlandt in his 'Physiological Plant Anatomy' attributes to Tyndall the theory that the secretion of certain oils, produced exogenously, gives rise to a vapour screen around the plant and thus prevents an excessive rise of internal temperature under conditions of insolation, and so reduces ensuing water loss by transpiration. Excessive loss of heat by night under a clear sky is also prevented. The senior author has been unable to discover any such theory in Tyndall's memoirs, and Detto (1903), who reviews the earlier literature, states his inability to discover the origin of the theory. This author also raises various objections to the theory, the most weighty of which is due to Köppen. It is pointed out that a dense ethereal vapour screen, thick enough to bring about an appreciable absorption of radiant heat, is most improbable, except perhaps where the surrounding air is completely still, a condition extremely rarely attained in the open. In this respect, recent work by Ramsey, Butler, and Sang (1938) is pertinent. These workers found that even for rapidly transpiring leaves of *Rumex hydrolapathum* in a current of air of only 0.81 cm. per second a humidity gradient could not be demonstrated above the leaf surface at distances greater than 2 cm. It seems probable therefore that, even in very slowly moving air, ethereal vapour concentrations would fall off to very low values within a distance of 2 cm. from the leaf surface. Tyndall's experiments were carried out by comparing the heat absorption of air passed over filter-paper soaked in the oils or over dried plants, with that of pure air. The thickness of his absorbing layer was 4 ft. All his figures were expressed as ratios, but the absolute absorptions in percentage of the total incident radiation have been



calculated from his original data. In addition to the absorption of his 4 ft. vapour layer, calculations were made of the theoretical absorptions of layers 15 in. and 2 cm. thick. The relation between absorption and thickness of the absorbing layer was assumed to be that expressed by Lambert's Law, i.e.

$$I = I_0 e^{-kd},$$

where  $I$  and  $I_0$  represent transmitted and incident energies respectively,  $d$  is the thickness of the absorbing layer, and  $k$  is the absorption coefficient. The following table (Table I) shows the percentage absorption values for a representative selection of oils used by Tyndall.

TABLE I

Source of vapour.	Absorption relative to the same thickness of dry air.	Per cent. absorption by 4-ft. layer.	Per cent. absorption by 15-in. layer.	Per cent. absorption by 2 cm. layer.
<i>Oils.</i>				
Lavender . . .	60	4.72	1.48	0.080
Lemon . . .	65	5.12	1.64	0.086
Rosemary . . .	74	5.82	1.86	0.097
Anise . . .	372	29.3	8.96	0.349
<i>Dried Plants.</i>				
Thyme . . .	33	2.60	0.863	0.043
Peppermint . . .	34	2.68	0.890	0.045
Lavender . . .	32	2.53	0.830	0.041

It was the very high values of the absorption ratios relative to air that struck the originators of the theory, but no one seems to have attempted to estimate the actual energy absorption by the oils. It will be seen from these derived figures that only screens of saturated vapour of very great thickness could bring about an appreciable reduction in the total energy incident on the leaf surface. From the last column it will be seen that screens of the exogenous oils 2 cm. thick give values in the region of a tenth to a twentieth per cent. It is this order of effect which would be expected in the field.

Once the idea had been formulated, other theories, suggesting a function for ethereal oils in the water balance and temperature relationships of plants, were evolved and duly suggested by physiologists. Thus Dixon (1914) found that the vapours streaming from the twigs of *Artemisia absinthium* brought about a reduction in the transpiration water-loss of leafy shoots of *Cytisus*. He was of the opinion that these vapours diffused into the leaf via the stomata and retarded the rate of water evaporation from the mesophyll cells, either by depressing the 'activity' of the protoplasm or by retarding the rate of diffusion of water vapour through the intercellular spaces. In the supposed action on protoplasmic 'activity' one is reminded of the action of anaesthetics in bringing about a change in the permeability of the cell membrane to water. Such

an action on the guard cells might easily result in closure and consequent reduction in water loss. Detto (1903) also found that menthol vapour depressed the rate of water loss from twigs of *Syringa*, but this was accompanied by death of a large area of leaf surface. These and similar results with other oils and other plants led him to the conclusion that ethereal oils played no part in regulating water loss in the field.

It must be borne in mind that a reduction in total incident energy falling on the leaf may not result in a corresponding decrease in leaf temperature. Indeed, if there is an accompanying physiological effect of the oils bringing about a decrease in the rate of water loss from the leaves, the internal temperature may rise in the presence of the vapour. Bearing in mind from previous considerations the very small absorption of incident energy expected, any large reduction in the rate of evaporation of water would result in an excessive increase in leaf temperature on insolation, a conclusion apparently not appreciated by the early workers.

Considered from a purely physical standpoint the rate of evaporation from a porous surface such as a cell wall should not be affected to any measurable extent by the presence in the gas above it of small concentrations of oil molecules. If, however, the oil could form a monomolecular layer partially or completely covering the water surfaces in the cell wall, the rate of evaporation would be considerably reduced. Under these conditions the exchange of  $\text{CO}_2$  and  $\text{O}_2$  might also be considerably impaired.

Another role suggested for these oils comes from Grisebach, as reported by Detto. He was of the opinion that the evaporation of oils from the surface of the leaf might supplement the cooling effect of transpiration. It seems unlikely that this evaporation could go on at a sufficiently high and steady rate to provide any appreciable cooling effect.

Thus we see that, as a result of an early uncritical attempt to attribute a biological significance to Tyndall's observations, a number of theories have sprung up, suggesting that the function of ethereal oils is to regulate the water loss, in bright sunlight, of those plants secreting them. This, of course, could only apply to those oils which can evaporate from the leaf surface and diffuse into the intercellular spaces of the leaf, e.g. those produced exogenously and those of a few species possessing internal oil sacs with opening mechanisms (*Ruta*, *Eucalyptus*). Except for the few meagre experiments of Dixon and Detto, no attempts have been made to verify these theories and the idea has been perpetuated. The experiments to be described are an attempt to determine once and for all the factual basis for these theories.

#### EXPERIMENTS ON THE IRRADIATION OF LEAVES THROUGH THICK LAYERS OF AIR SATURATED WITH VAPOURS OF ETHEREAL OILS

The first type of experiment performed was a direct attempt to verify the initial theory of the effect of vapour screens on leaf temperatures. Since these

possible effects of oil vapours are presumably not specific to the plants secreting them, it should be possible to evoke the effects in any species of leaf. A species not producing oils of its own is preferable for experimentation, since this avoids complications which might arise from the added action of the oils secreted by the plant itself. *Prunus laurocerasus* was chosen for the investigations since a great deal is known of its general physiological behaviour.

The apparatus used is shown in Fig. 1. A single leaf of a small potted bush was sealed with plastic wax into a cylindrical glass chamber (Ch) 16 in. long. The leaf was irradiated by a 1,000-watt lamp, the radiations from which were concentrated by the large condenser c. The lamp was kept cool during the illumination by immersion in running water. The temperature difference between the leaf lamina and the air behind the leaf was determined by a thermocouple H, one end of which was in the form of a needle which could be easily introduced into the leaf lamina. Use of a sensitive mirror galvanometer made differences of temperature of  $1/500^{\circ}$  C. easily detectable. A mercury thermometer recorded air temperatures. Throughout the course of an experiment air was circulated throughout the chamber at a constant rate of 25 litres per hour by the small electrically driven rotary pump P. The total volume of the circuit was approximately 5 litres, and this rate of circulation of air thus represents a linear rate of flow in the chamber of 3.5 cm. per minute. The circulating air was dried by passing through a large tube of  $\text{CaCl}_2$ . Air temperatures were maintained approximately constant by passing through the glass coil G immersed in a tank of water. Carbon dioxide was removed from the air by a soda-lime tube.

At the start of an experiment pure air was circulated, and temperature differences followed in the darkness by covering the chamber with black velvet. After a period of about an hour the leaf was irradiated and temperature differences again followed in pure air until a steady state seemed to have been reached. At this point, by suitable adjustment of the two taps T the bottle E, containing cotton-wool soaked in the oil under investigation, was introduced into the closed circuit. In this way the rapid saturation of the circulating air by the vapour was ensured. Temperature differences in this vapour were followed for two hours, after which time taps were again adjusted to isolate bottle E and allow pure air to sweep through the chamber. After a further period of two hours the lamp was switched off and readings again taken with the leaf in darkness.

The exogenous oils studied were rosemary, thyme, verbena, lavender, and peppermint. In addition three other oils were investigated. They were anise, eucalyptus, and lemon oils, the first being an obvious choice because of its extremely high absorptive capacity.

In Fig. 2 typical results for each of the oils used are shown in graphical form. At the beginning of the circulation of dry air, leaf temperatures sank rapidly below air temperatures, due presumably to the increase in transpiration rate under these conditions. Illumination resulted in a rapid increase in

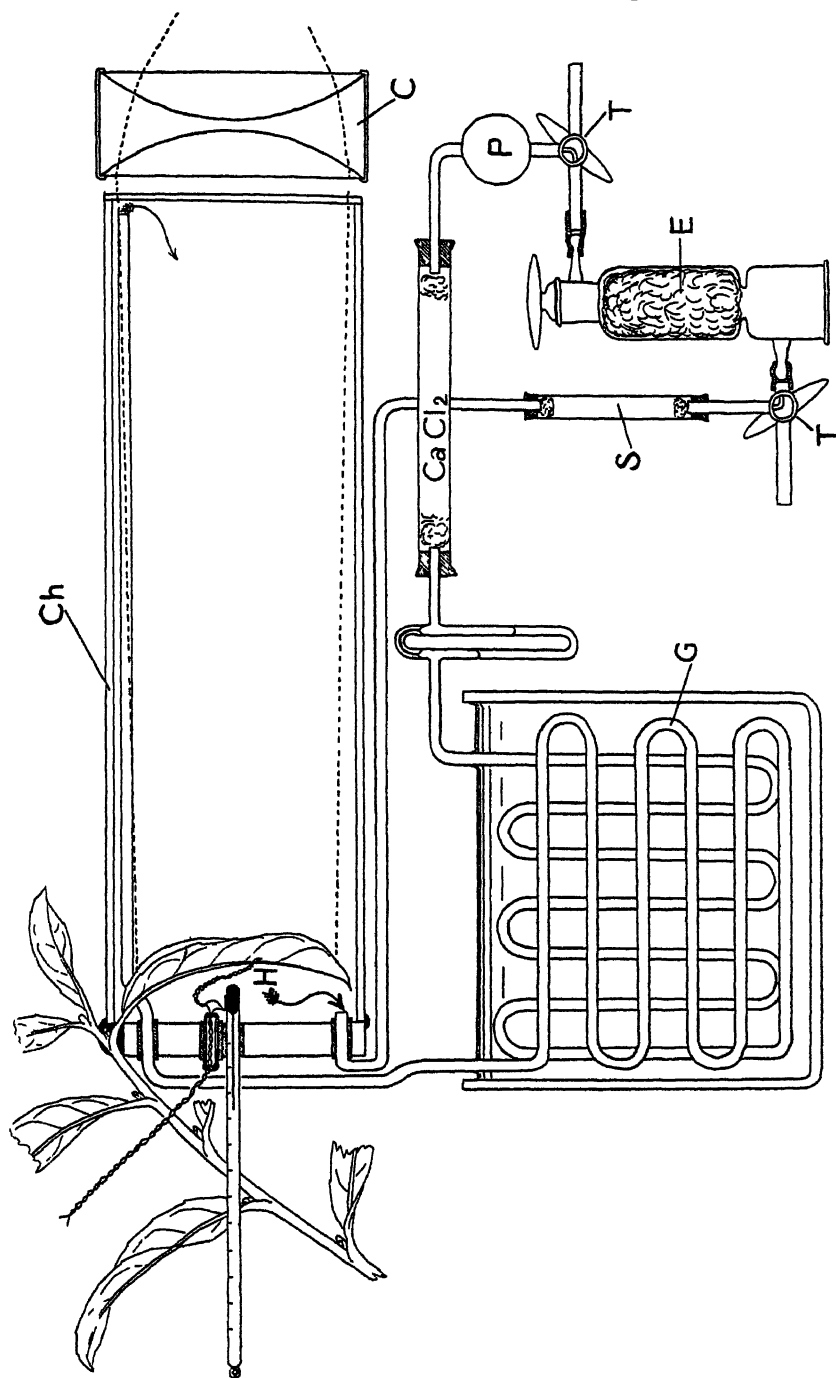


FIG. 1. Apparatus for investigating the effects of thick screens of air saturated with the vapours of ethereal oils on the temperatures of insulated leaves. (For explanation see text.)

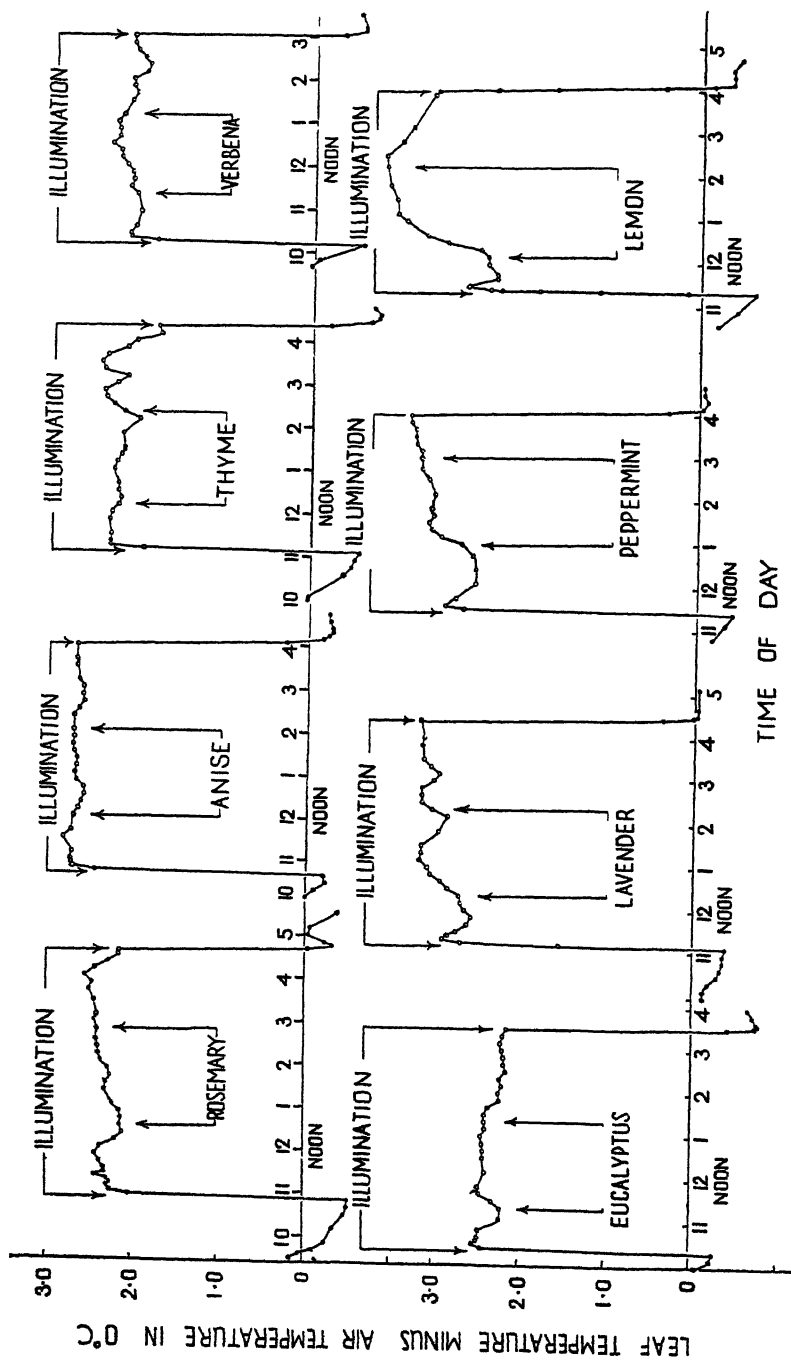


FIG. 2. Graphs of eight experiments showing the effects on the temperature differences between leaf and surrounding air of illumination through a 15-in. screen of pure air and one of air saturated with the vapours of various ethereal oils. The periods of illumination and of treatment with oil vapours are shown by the pairs of vertical arrows.

leaf temperatures to a maximum in 10–15 minutes, followed in some cases by a fall. This fall is due presumably to a further increase in transpiration rate on illumination. Later results suggest that this is brought about, to a large extent, by stomatal opening. Careful examination of the graphs show that in the case of anise and thyme oils no significant effects of the vapour screens on temperature differences can be detected. In all other cases, however, an increase seems to occur on the introduction of the oil. This is scarcely significant in the case of rosemary, verbena, and eucalyptus oils, but much larger with lavender, peppermint, and lemon oils. This increase can be brought about only by a decrease in the transpiration rate, since all other air conditions remained constant throughout the experiments. In three cases (eucalyptus, verbena, and lemon) there is a suggestion that removal of the vapour screen resulted in an increase in the transpiration to its previous rate, since temperature differences again declined. This small effect of these oil vapours is similar to the effects recorded by Dixon and Detto. The treatment with lemon oil resulted in death and subsequent browning of large areas of the leaf, a condition probably largely responsible for the depression of the transpiration. It is probable that the effects with eucalyptus and verbena oils were due to a similar toxic action on the leaf, although no visible browning was observed. On the other hand, rosemary oil, which produced a small effect, brought about browning of the leaf after prolonged exposures (24 hours).

It is conceivable, however, that the oil vapours produced a reduction in the total energy incident on the leaf surface, but that the effect of this on the leaf temperatures was very much overshadowed by an opposing effect arising from a depression of the transpiration rate. In the case of anise and thyme oils, for example, these two effects might have been equal and opposite, giving no significant change in leaf temperatures. The following series of experiments were performed to throw light on this point.

#### THE EFFECT OF OIL VAPOURS ON THE TRANSPIRATION RATE AND STOMATAL RESISTANCE

In this series of experiments freshly cut shoots of cherry laurel, possessing eight to ten leaves arranged more or less in one plane, were employed. These shoots were sealed into the flat glass chamber *c*, shown in Fig. 3, the lid being sealed with low melting-point luting wax. The cut end of the shoot was inserted into a potometer, after taking the usual precautions to prevent air blocks in the xylem. The rate of water absorption by the shoot was taken as a direct measure of the transpiration rate. Temperature differences between the leaf and the air were measured as in the previous experiments. In a large number of experiments continuous readings were taken of the resistance to mass flow of air of the stomata over a small area of one of the leaves using a resistance porometer as described by Gregory and Pearse (1934). A small porometer cup *P* was used, the join between the leaf surface and the cup being

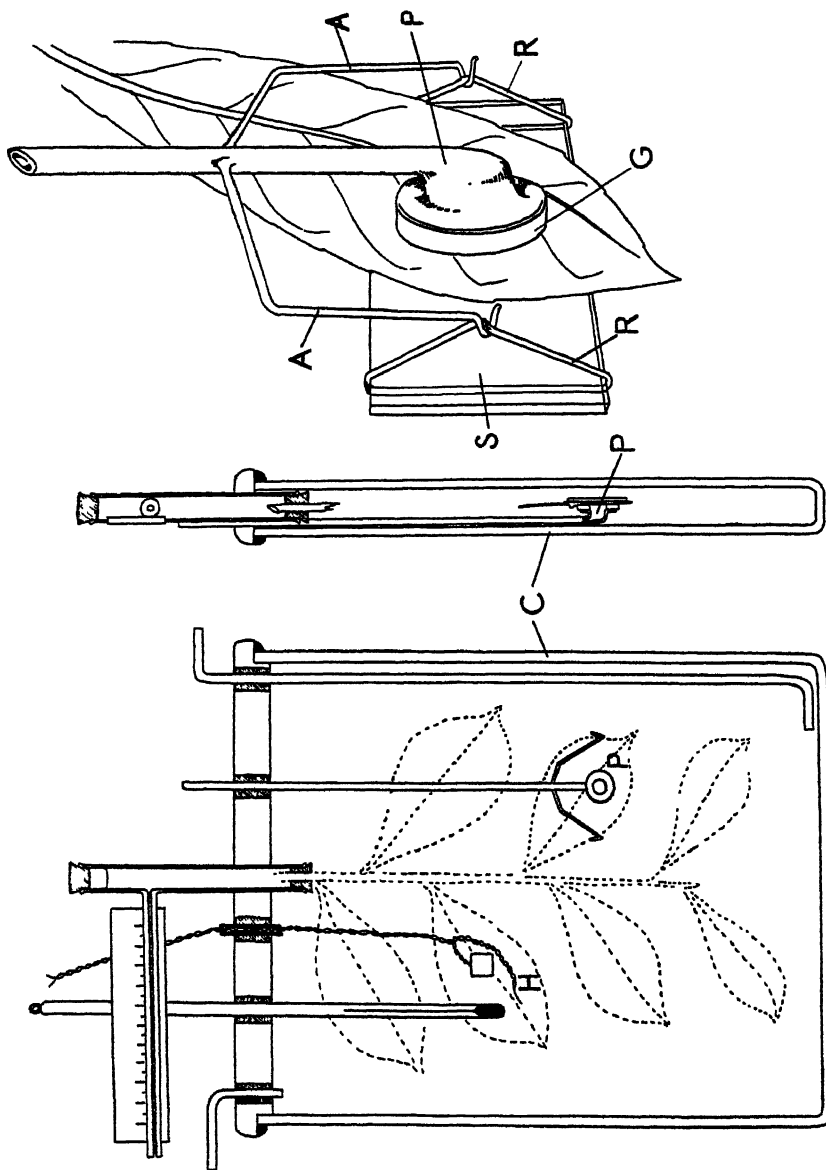


FIG. 3. Apparatus for investigating the effects of air saturated with the vapours of ethereal oils on transpiration rate, resistance of stomata to mass air flow, and leaf temperatures under various light intensity conditions. (For explanation see text.)

made by a small 30 per cent. gelatine washer G. The cup, washer, and leaf were kept firmly in contact by the microscope slide s hooked to the lateral arms A by the rubber bands R. Circulation of air and of oil vapour through the chamber was carried out as in the previous experiments. Here the saturated vapour screen was only 1.5 cm. thick, and thus approximated to conditions which presumably exist in nature. The chamber was immersed

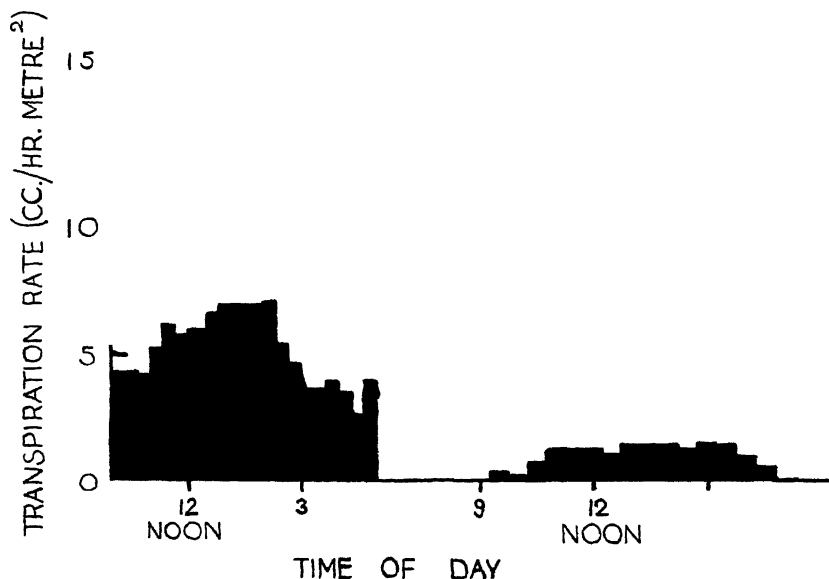


FIG. 4. Graphs of two experiments showing daily transpiration drifts of shoots in a current of dry air and in low light intensities.

in a large glass tank of water, kept at a constant temperature over the course of an experiment. Experiments were carried out in the subdued light of the laboratory, where the light intensities never rose above 50 lux, and in the radiations produced by a 1,000-watt lamp at 21 cm. from the chamber. This latter intensity, as measured by a photo-electric meter, was 14,500 lux.

Several experiments were carried out to determine the normal behaviour of leaves under these conditions. Fig. 4 shows the transpiration drift of two shoots in low light intensities throughout the day. Although there is a considerable difference in the average transpiration intensity of these two shoots, both show the same shape of curve with its upward drift during the morning, reaching a maximum at about 2 p.m., and the subsequent decline in the afternoon. It is suggested that this may be due to changes in stomatal aperture which are either due to an intrinsic rhythm or to changes in low light intensity of the laboratory throughout the day.

Fig. 5 shows the effect of high light intensities of illumination on the transpiration rate. The black areas represent transpiration rates in low light intensities and the white areas rates in high light intensities. In each case high



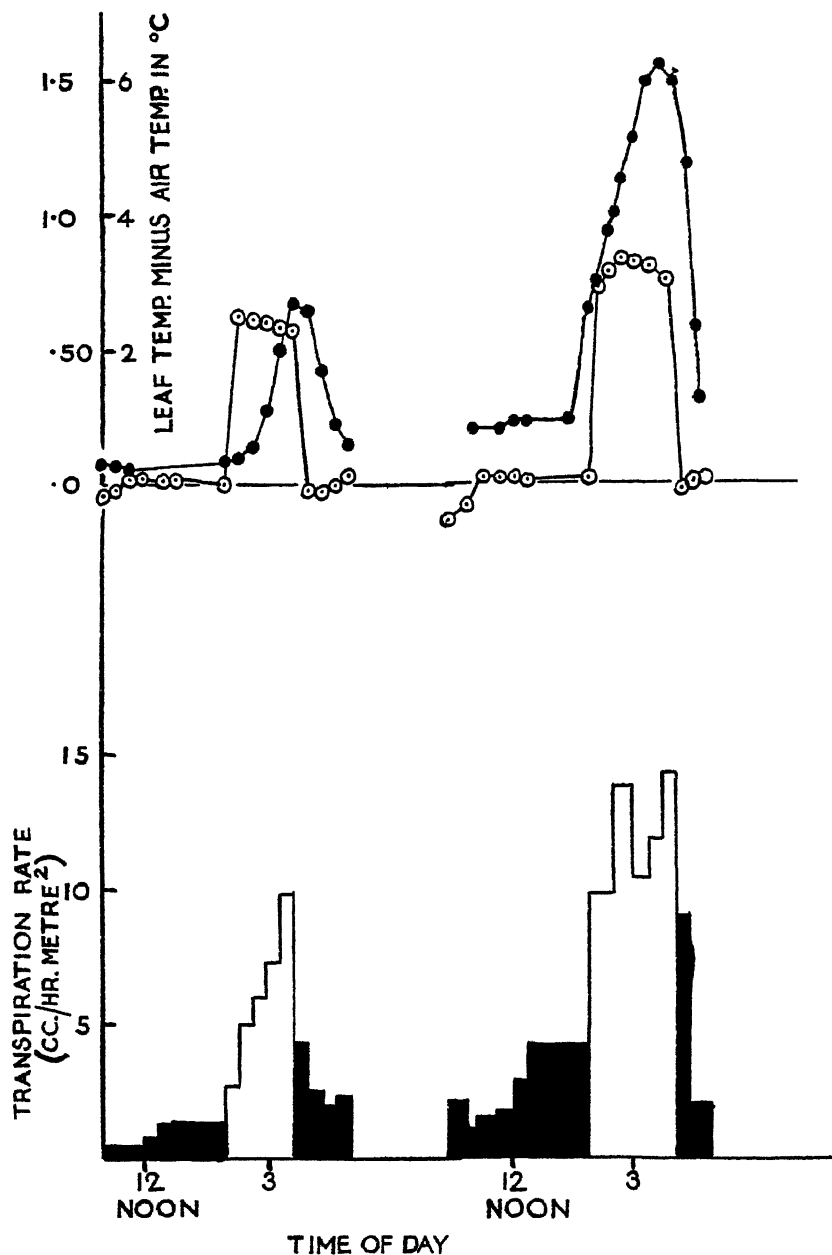


FIG. 5. The lower graphs showing two experiments on the effects of high light intensities on the transpiration drift of shoots in a current of dry air throughout the day. The black areas represent results in low light intensities and the white areas results in high light intensities. The upper graphs show the corresponding drifts in temperature difference between leaf and air (ringed points) and in the reciprocal of the square root of the stomatal resistance (R) (black points).

light intensities are seen to bring about a considerable increase in transpiration rate and these are closely correlated with decreases in stomatal resistance. It seems likely that here the action of the light on the transpiration rate is due very largely to its action on the stomata.

Three oils were used in this series of experiments, i.e. rosemary, thyme,

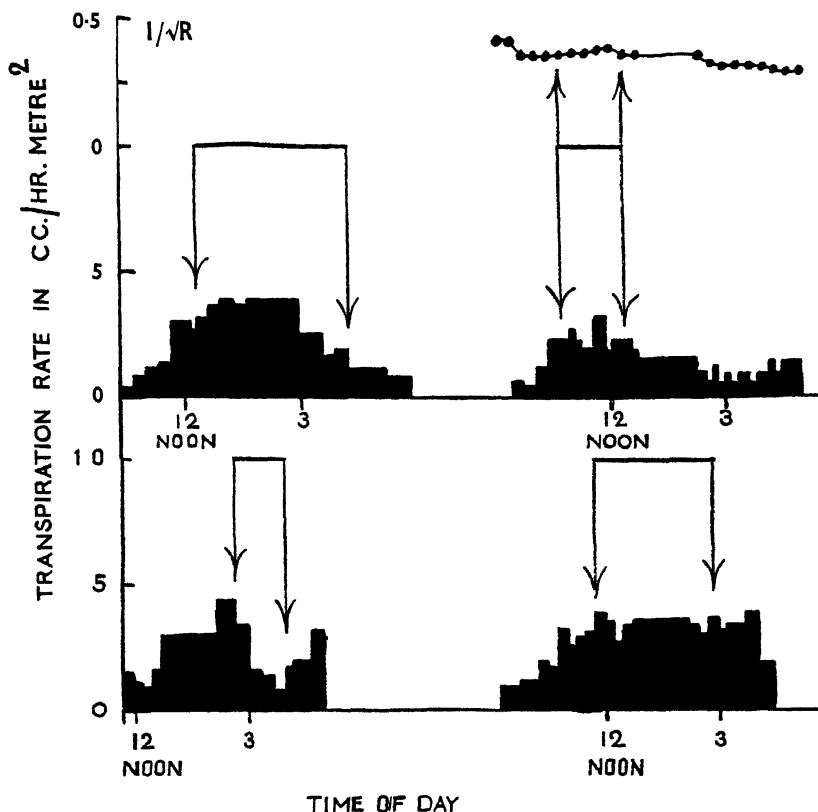


FIG. 6. Graphs of four experiments showing the effects of varying periods of exposure to air saturated with the vapour of rosemary oil on the transpiration drift in low light intensities. The pairs of vertical arrows mark the periods.

and anise. The last is produced only in internal ducts in seeds, and therefore any effect it may be shown to have on transpiration rates or leaf temperatures can have no direct ecological significance. It was chosen for experimentation solely because of its apparently high absorption capacity for radiant heat.

The effects of periods of rosemary oil vapour on the transpiration rates in low light intensities are shown in Fig. 6. The periods of exposure to the vapour are shown by the pairs of vertical arrows. It will be seen that these drifts are essentially the same as the normal ones in Fig. 4, and no effect of the vapour can be detected. In one case a curve of stomatal resistance is also shown, and here, too, no effect of the vapour can be seen. In Fig. 7 a number

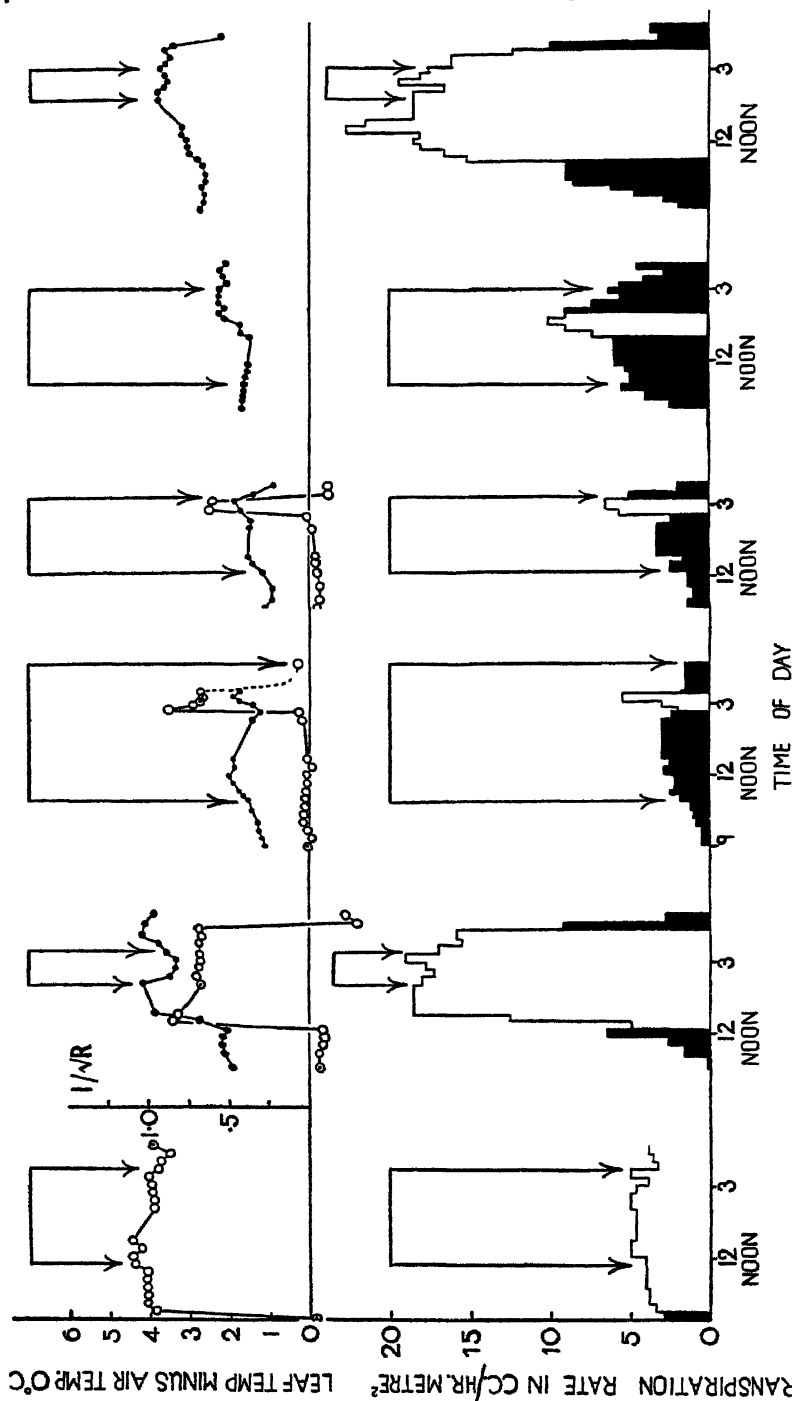


FIG. 7. Graphs of six experiments showing the effects of varying periods of exposure to the vapour of rosemary oil on the rate of transpiration (lower stepped curves), temperature differences between leaf and air (ringed points), and the reciprocal of the square root of the stomatal resistance (black points), in both high and low light intensities. The results are expressed as in Figs. 4-6.

of experimental results are collected showing the effects of varying periods of exposure to rosemary oil vapour both in low and in high light intensities. The upper curves are for temperature differences between leaf and air (ringed points) and for  $1/\sqrt{R}$ , where  $R$  is the resistance of the stomata to mass flow of air in arbitrary units (black points). The lower curves are for the corresponding transpiration drifts. With the possible exception of the first graph,

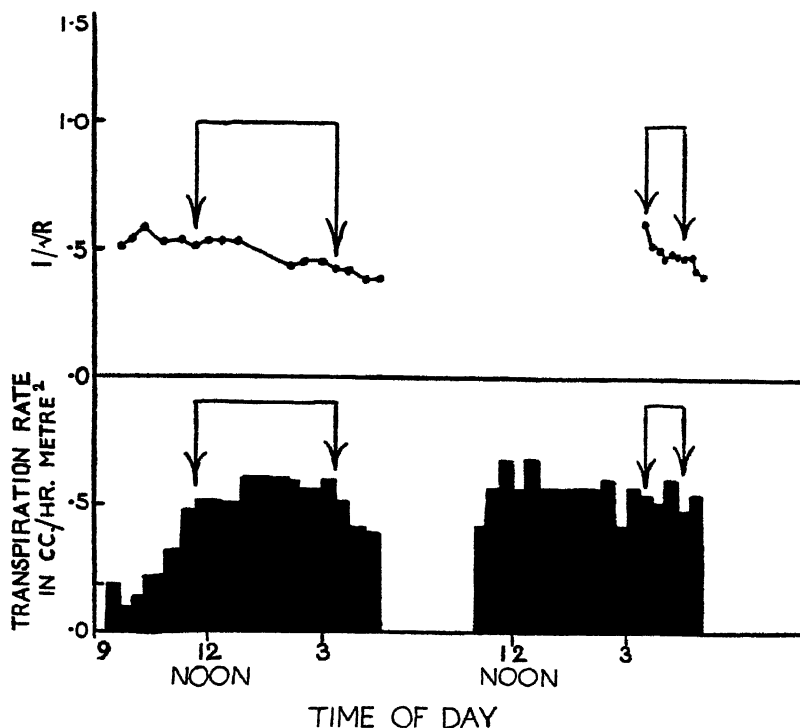


FIG. 8. Graphs of two experiments showing the effects of varying periods of exposure to the vapour of thyme oil on the transpiration drift and the reciprocal of the square root of the stomatal resistance ( $R$ ) in low light intensity. The pairs of vertical arrow mark the periods.

illumination with high light intensities resulted in a considerable but gradual increase in the transpiration rate. Here, as in the results of Fig. 5, there is an associated decrease in the stomatal resistance, which is, however, of much smaller magnitude. With the exception of the second set of results, all the graphs show that periods of exposure to the saturated vapour of the oil had no significant effects on the leaf temperatures, stomatal apertures, or transpiration rates. In the second experiment rosemary oil vapour seems to effect a transitory closing of the stomata, with which is associated a small decrease in the transpiration rate.

Only two experiments were carried out with thyme oil, and these were in low light intensities. In Fig. 8 the corresponding drifts of the transpiration

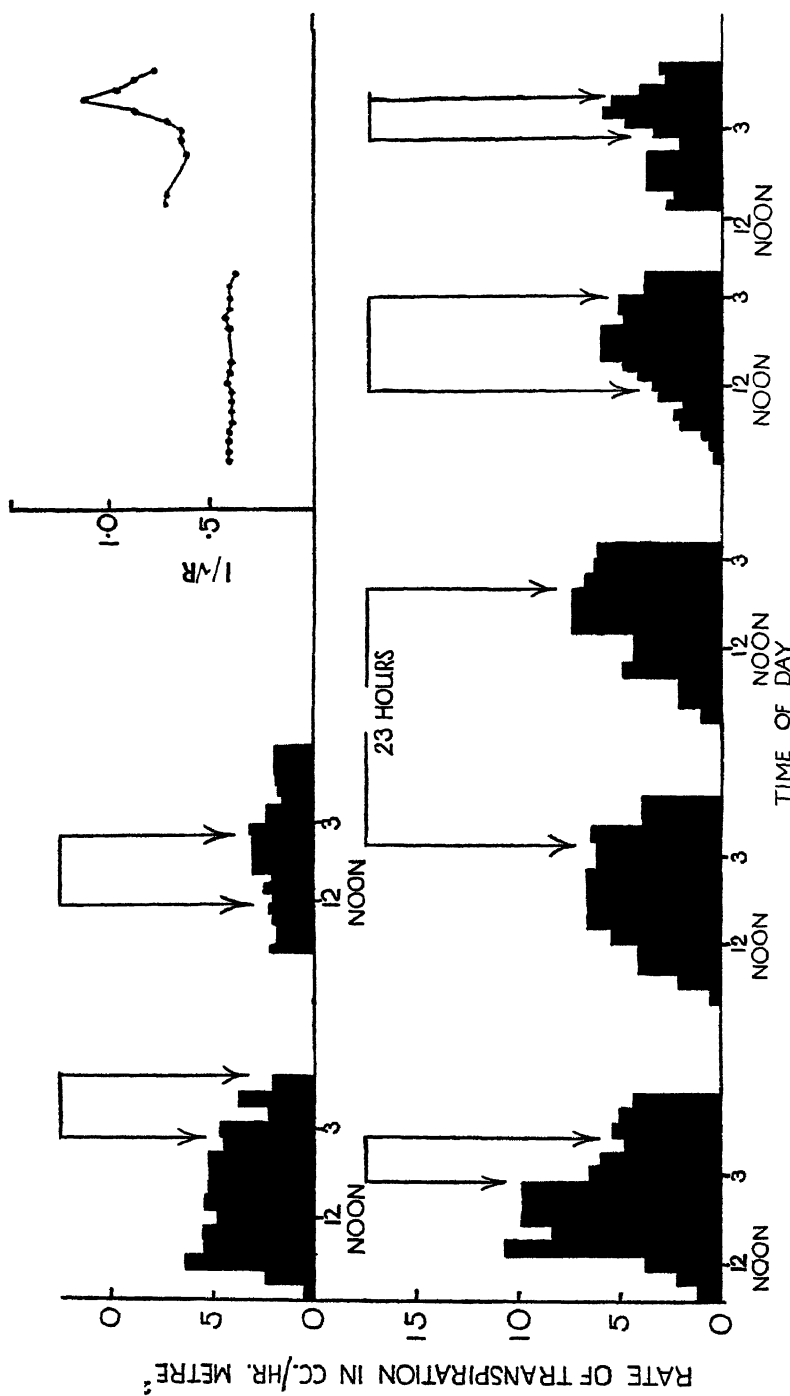


FIG. 9. Graphs of seven experiments showing the effects of varying periods of exposure to the vapour of anise oil on the transpiration drift and the reciprocal of the stomatal resistance ( $R$ ) in low light intensity. The pairs of vertical arrows mark the periods.

and stomatal resistances are shown. It seems that in both these experiments exposure to the vapour resulted in a slight closing of the stomata, but there is no apparent effect on the transpiration.

Experiments with anise oil vapour were carried out in both high and low

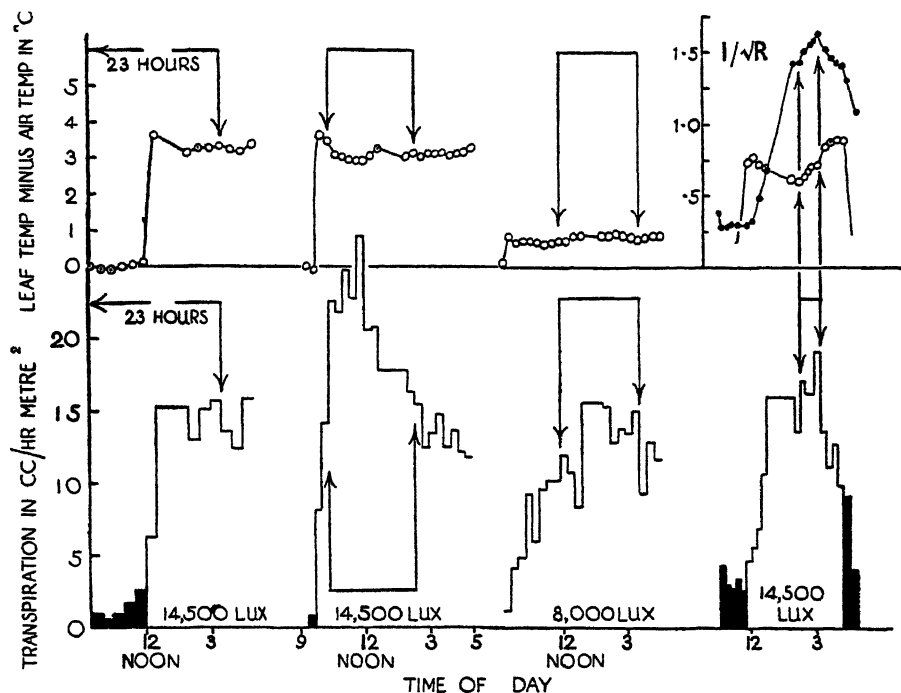


FIG. 10. Graphs of four experiments showing the effects of varying periods of exposure to the vapour of anise oil on the rates of transpiration, the difference of temperature between leaf and air, and the reciprocal of the square root of the stomatal resistance ( $R$ ) in two high light intensities. (Results are expressed as in Figs. 4-9.)

light intensities. The results of a number of such experiments are collected in Figs. 9 and 10. Here again no trace of an effect of the vapour can be detected, even in the case of those shoots which had been exposed for twenty-three hours to the saturated vapour.

These three sets of experiments all demonstrate conclusively that air saturated with the vapours of these oils passed over the leaves of cherry laurel for considerable periods had no significant effects on the rate of transpiration, the leaf temperatures in high intensities, or on the stomatal resistance.

#### OIL EVAPORATION AND LEAF TEMPERATURES

The theory of Grisebach mentioned in the introduction still remains unchecked. It was that the unilateral evaporation of ethereal oils from the leaf might augment very considerably the cooling effect of the transpiration under

conditions of insulation. In order to assess the plausibility of this theory experiments were carried out on the evaporation of a number of oils under constant conditions. A wide glass tube G (see Fig. 11) was fitted with two bungs, through which passed inlet and outlet tubes and a mercury thermometer. A small glass hook (not shown in the figure) was fixed into one bung so that a small roll of filter-paper (6 cm. long by 1.6 cm. in diameter) could be

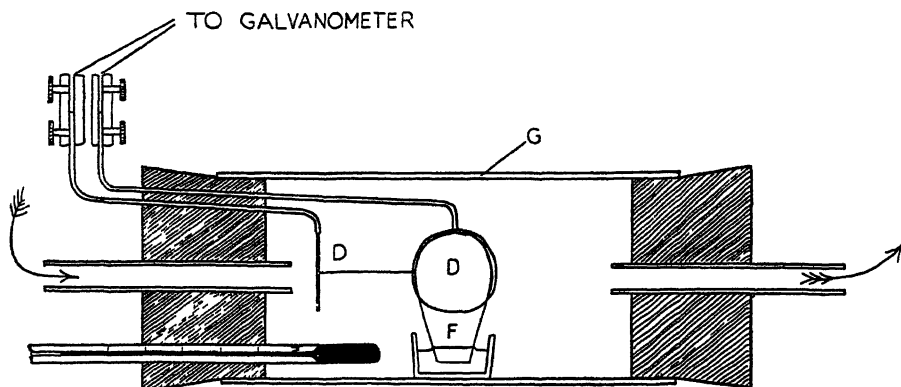


FIG. 11. Chamber used in the investigations on the rates of evaporation of ethereal oils from filter-paper into a pure air-stream, and on the associated cooling effects. (For explanation see text.)

suspended from it by fine copper wire. The roll of paper was soaked in the oil to be studied and then suspended in the middle of the chamber. A current of pure dry air was then passed over the roll at a constant rate indicated by a capillary flow-meter in the circuit. From this the calculated linear rate of air flow in the chamber was 0.35 cm. per second. The temperature in the chamber during an experiment was kept constant to within a degree. The chamber was also darkened during an experiment. The roll of paper was carefully removed at intervals and rapidly weighed to 0.1 mg. From the losses in weight the rates of evaporation of each oil from the filter-paper were determined and plotted on a graph. Results for the five exogenous oils rosemary, thyme, peppermint, lavender, and verbenia are shown in Fig. 12. A result for the evaporation of water into the moist air of the laboratory (R.H. = 70 per cent. approx.) under the same conditions of temperature and air flow is also shown. In all cases there is a progressive decrease in the rate as the paper dries out, and the decrease is more rapid, as would be expected, in the case of the more volatile oils. Since the roll of paper was completely saturated at the start of the experiment, it is fairly safe to conclude that the initial rates approximate to the rates of evaporation of these oils from a free liquid surface. It should also be noted that since these oils were evaporating into pure air the rates correspond to a saturation deficit of 100 per cent. for the vapour. Even so, these rates are all very much smaller than that of water evaporating into an atmosphere with a saturation deficit of only 30 per cent.

In addition to these experiments on rates of vaporization, determinations have also been made of the differences of temperature between liquid and air arising as a result of the evaporation. To do this two small copper discs (D in Fig. 11) 1.5 cm. in diameter were soldered to two copper leads passing through the bung, and arranged in the positions shown in the diagram. These discs

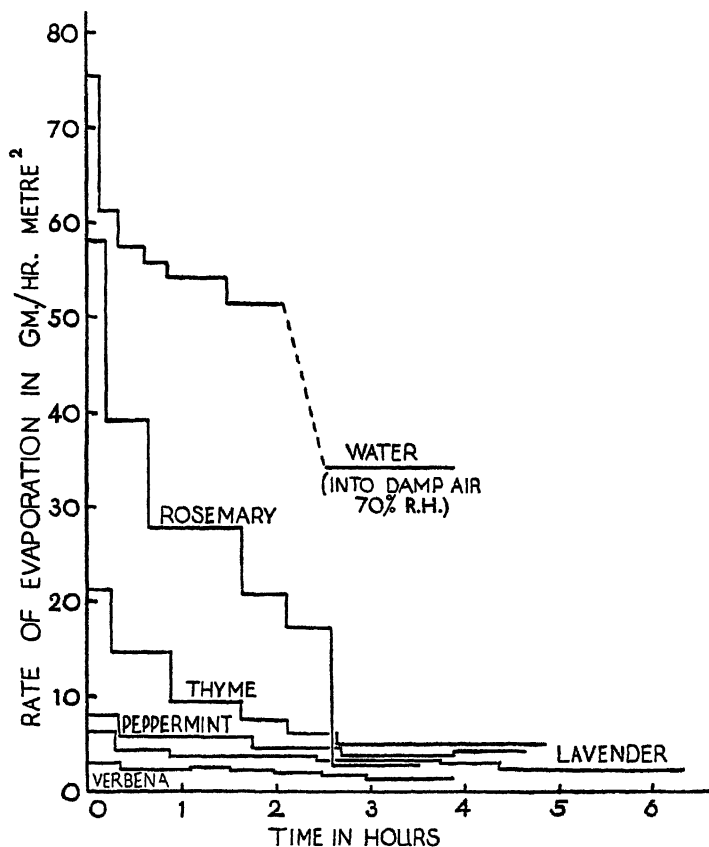


FIG. 12. Graphs showing the courses of evaporation from filter-paper of five exogenous ethereal oils into a pure air current of 0.35 cm. per second. A curve for water evaporating into damp air is given for comparison.

were connected by a fine wire of constantan, thus making a thermocouple. Two small tongues of filter-paper F were then cut and soaked in the oil to be studied. They were then placed one on each side of the central disc, where they remained fixed by capillary forces. The lower ends of the two tongues, which were in contact, dipped into a small glazed porcelain dish filled with the oil. Air was circulated at the same rate as before in a direction shown by the arrow and the temperature differences between the two discs measured by connecting the leads to a galvanometer previously calibrated for known



temperature differences. Under these conditions of air flow of 0.35 cm. per second steady temperature differences were attained in the course of a minute or so. The values obtained are recorded in Table II, together with that for the evaporation of water into a moist air stream.

TABLE II

Liquid.	Air temperature in °C.	Cooling produced by evaporation in °C.		Number of observations.
		Mean.	Standard Error.	
Rosemary oil . . .	15.5	0.77	0.035	5
Lavender oil . . .	15.9	0.24	0.022	8
Verbena oil . . .	14.1	0.10	0.002	6
" . . .	16.7	0.18	0.026	6
Thyme oil . . .	17.3	0.21	0.016	9
Peppermint oil . . .	15.1	0.13	0.014	12
Water . . .	15.6	2.68	0.07	7
(R.H. 70 per cent.)				

These values for the temperature differences give the order of the maximum degree of cooling for evaporation of the oils from a free surface and are undoubtedly very much larger than could arise by evaporation of the oils from leaves in nature. Simple calculations show that for a degree of cooling of only 0.1° C. leaves would have to lose oil at rates of the order of 1-2 per cent. of their fresh weight per hour. These few figures serve to show what a very insignificant part evaporation of these oils can play in the cooling of the plant under conditions of insolation.

### CONCLUSIONS

The results of the foregoing investigations make it extremely unlikely that the vapours of ethereal oils serve any ecological function in regulating, directly or indirectly, either the temperatures in strong sunlight or the rates of transpiration of the leaves producing the oils. It is much more probable that this oil production by plants accustomed to growth under dry conditions is merely an outcome of a peculiar metabolism bound up in some way with growth conditions. Presumably a similar relationship holds for the production of mucilages by the desert succulent. The solution of the problem of the significance of ethereal oil production lies therefore in researches into the metabolism of the plants concerned. Our lack of knowledge on this point therefore makes further theorizing premature.

### SUMMARY

1. Theoretical considerations have shown that vapour screens of ethereal oils around leaves can absorb at most only a fraction of a per cent. of the total incident heat radiations.

2. Comparison of leaf temperatures of the cherry laurel on irradiation through 15-in. screens of pure dry air and of dry air saturated with the vapours of a number of essential oils showed that differences between leaf and air temperatures tended to increase rather than decrease in the presence of the vapour screen. This is presumably due to transpiration depression arising from a toxic action of the vapours. The oils used were anise, rosemary, thyme, verbena, peppermint, lemon, eucalyptus, and lavender.

3. Exposure of shoots of cherry laurel to thin 1.5 cm. screens of air saturated with vapours of rosemary, thyme, and anise oils in both high and low light intensities failed to produce any measurable effect on either the transpiration rate, leaf temperatures, or stomatal resistance.

4. Experiments on rates of evaporation of oils from filter-paper and the associated cooling effects demonstrated conclusively that the latter are too small to have any ecological significance.

5. It is concluded that in the plants which produce them ethereal oils play no part in regulating water loss or leaf temperatures.

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# Growth of *Chlorella* in Relation to Light Intensity

BY

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AND

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With two Figures in the Text

**T**HIS paper deals with the effects of different light intensities upon the growth of the alga *Chlorella*, the strain used being that employed by Hopkins and Wann (1926) and also in earlier work by Pearsall and Loose (1937). The alga is grown in glucose media, although the results are not directly comparable with those of Bristol Roach (1928), in which a similar alga was grown both in mineral media and in glucose media.

## METHODS

In the present investigation the alga is grown in conical Pyrex flasks of 230 c.c. capacity on 50 c.c. of a medium composed of equal parts of solutions A and B with the addition of 2 c.c. of distilled water to counteract loss of water on sterilization.

Solution A: 20 gm. of glucose, 0.4 gm.  $\text{MgSO}_4$ , 0.4 gm. sodium citrate, 1.5 gm. ammonium nitrate, and a trace of ferrous sulphate per litre.

Solution B: 14.528 gm.  $\text{KH}_2\text{PO}_4$  and 4.646 gm.  $\text{K}_2\text{HPO}_4$  per litre. This gives pH 6.0.

The flasks were closed with cotton-wool plugs and sterilized by autoclaving for fifteen minutes at 11 lb. pressure. They were inoculated aseptically from a previous culture growing exponentially and then placed on a water-bath, so designed that the bottom cm. of each flask projected below the support and was bathed by circulating water kept at  $23^\circ \pm 0.2^\circ \text{C}$ . by a thermostat. The variation of temperature between different flasks at the same time was less than  $0.1^\circ \text{C}$ . The room temperature was maintained at  $20\text{--}21^\circ \text{C}$ . The flasks were arranged on circular arcs at fixed distances from the light, spacing being such that flasks were not shaded from direct light. Light intensities were measured, with the flasks in position, by a calibrated Weston photo-voltaic cell. The light intensity was that on the horizontal face of the cell in the position usually occupied by the algal film at the bottom of the culture flask. The source of light was a 100-watt 'daylight' lamp cooled by a distilled water screen circulating from an automatic siphon reservoir. No consistent

temperature difference between the most strongly illuminated flasks and those farthest from the light could be detected. The cultures were shaken morning and evening in a constant manner.

The technique of estimating the cell numbers has been described in earlier papers (Pearsall and Loose, 1937). It was done with a Fuch's-Rosenthal haemocytometer, counting a minimum of 250 cells in each of three different drops of the culture medium.

We are indebted to the Leverhulme Research Trust for a grant in aid of this culture work.

#### DETAILS OF EXPERIMENTS

The results described in the following pages and in Table I are those from a single experiment which is typical of others, five having been carried out at

TABLE I  
*Cell Number (per c.mm.) in Relation to Light Intensity*

Series . . . Light intensity (10 <sup>2</sup> m.c.) . Days.	A.	B.	C.	D.
	12	4.8	2.6	0.2
0	18	18	18	18
2	194	—	184	180
3	424	412	416	410
4	1,610	1,322	1,290	1,200
5	5,400	3,650	4,008	—
6	11,950	9,350	8,500	6,050
7	23,675	16,470	16,222	12,100
8	32,675	25,033	19,897	16,742
9	36,775	26,517	23,287	19,270
10	43,545	31,153	25,087	21,560
11	46,800	35,317	28,942	23,985
12	49,965	40,475	32,725	26,187
14	51,380	45,775	38,750	30,400
15	57,000	46,140	39,150	32,265
16	57,450	47,280	38,375	33,150

different times. The remainder are less satisfactory, either because of less certain temperature control in high light intensity or because of unsuitable ranges of light conditions or because the latter part of the growth curve was not complete. In the present series four light intensities were employed, called A, B, C, and D, and respectively averaging 1,200, 480, 260, and 20 metre candles. It may be noted that within the range 30–10 m.c. no significant alterations in growth rate occurs. There were five or six cultures at each light intensity and the figures given for the cell numbers are usually four culture averages. These should give estimates within 700 of a large average when the cell number exceeds 20,000 per c.mm., Pearsall and Bengry (1940). In the present series, between 5,000 and 20,000 cells per c.mm., there is no instance when the individual replicate cultures differ from the mean by more than 1,000. In series A, however, only three cultures were possible with the

bath arrangements available. These were, however, very uniform, and the extreme variation from the mean was only  $\pm 400$  on an average, though it rose to  $\pm 1,100$  on day 7.

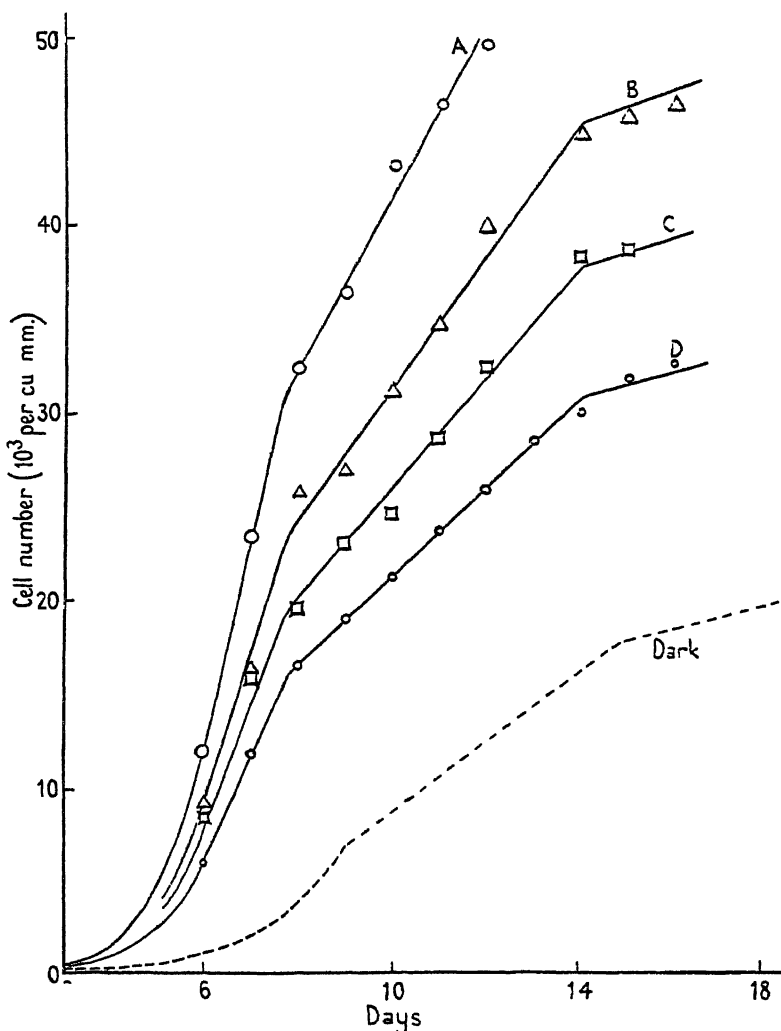


FIG. 1. Cell numbers in relation to time for four different light intensities: A, B, C and D. The lines drawn are 'standard' curves, adjusted as to level by rate factor (see text). The broken line is a typical growth curve in darkness, starting from a different inoculum.

#### ANALYSIS OF RESULTS

The data given in Table I and Fig. 1 show quite clearly that increasing the light intensity increases the rate of growth. Some further analysis of the results is desirable and a description of the methods of analysis used may now be given. The growth cycles are divisible into two main parts, a period of

exponential or logarithmic growth, terminating when the cell number reaches approximately 6,000 cells per c.mm., and a post-exponential period in which growth tends to be linear in relation to time.

The rates of growth during the exponential period are best compared on a logarithmic basis, the logarithmic growth rate  $k$  being obtained from  $\log N_t - \log N_0 = kt$ , where  $N_t$  and  $N_0$  are respectively the cell numbers at time  $t$  and initially. The values of  $k$  are given in Table II and discussed later.

The post-exponential phase starts about day 6 in the present results and can be subdivided into two periods. It is assumed that during both these the growth tends to be linear. The first post-exponential period only lasts two days, however, and on the evidence available here may possibly be a transition before the second and more definitely linear phase is entered. In order to compare the rates of growth in the post-exponential periods, we adopt one of two procedures. The first of these is to estimate the mean daily growth rates in each light intensity during the second linear phase of growth, that is, between days 8 and 14. These mean growth rates are given in Table III as increases in cell number per day, together with their standard errors. The ratios of the values in comparison with that for the lowest light intensity are also given.

The disadvantage of this procedure is that it takes no account of the first post-exponential period. The number of readings in this period is small and variability is possibly at its greatest. For these reasons, we employ as a second procedure a method which is based on the fact that the various post-exponential curves obtained in this (and other) experiments are all of a uniform type, such that by dividing the numbers for any given light intensity by an appropriate factor ( $r$ ) figures which fit a standard curve are obtained. The factors to be employed are obtained in the present instance by dividing the numbers for the lowest light intensity into those (on the same days) for any other light intensity. This is done on every day for which records exist between days 6 and 14 and the average of the different estimates of the factor for that light intensity is taken as  $r$ . The average values of  $r$  obtained in this way are given in Table III. They are really objective estimates of rates of growth and also have the advantage of comparing these rates in all the post-exponential phases. It will be seen from the table that they give relative figures which are almost exactly the same as the ratios between the average cell number increases per day, between days 8 and 14 (Table III), whose origin has already been described.

These rate factors can be employed in another way. If the cell numbers for each of the light series A to D be divided by the appropriate rate factor, all the values so obtained will tend to lie on a standard curve. By averaging these values on each day we can get average points showing the usual form of this standard curve, and it is of interest to note that the means for the present series are almost exactly those for all available data. Further, by multiplying the average values by the rate factor for any light intensity required, we can

get the 'expected' or standard curve for that light intensity. The lines drawn in Fig. 1 are all standard curves of this sort and, as it may be seen, they fit the observed points well and are, in fact, not far removed from the curves of best 'fit'.

#### THE EFFECTS OF LIGHT INTENSITY ON THE TWO MAIN PHASES OF GROWTH

The values of  $k$ , the logarithmic growth rate, are given in Table II. The first values are means from values for individual cultures below a cell number of 5,000. It is apparent that altering the light intensity has very little effect on the exponential phase of growth, the differences being doubtfully significant in most cases. If the daily numbers are examined it will be seen that significant differences do not appear until exponential growth has almost ceased. For example, only on day 5 is series A significantly higher than B and C. For this reason we also give the estimates of  $k$  for the first four days in light in Table III. Series B, C, and D are almost precisely the same.

Table II also contains for comparison the value of  $k$  in darkness as determined by Pearsall and Bengry (1940). This figure is much lower (0.29 cfd. with 0.46) and it shows that a small amount of light gives almost all the light effect characteristic of the exponential phase.

TABLE II

#### *Effect of Light Intensity upon Exponential Growth*

Series . . .	A.	B.	C.	D.	Dark.
Light intensity (10 <sup>3</sup> m.c.) .	12	4.8	2.6	0.2	0
$k$ , mean	0.490	0.470	0.463	0.455	0.29
$k$ , days 0-4	0.490	0.467	0.465	0.460	—

The comparison of this work with that of Bristol Roach (1928) is not without interest. That worker used a similar alga, *Scenedesmus costulatus* var. *chlorelloides*, isolated from soil, and in glucose media also obtained a large effect of light on exponential growth, the value of  $k$  rising from 0.20-0.25 (dark) to 0.40 or 0.47 in low (1) and high (16) light intensities respectively. It will be noted that the light range was small and the effect of increasing light intensity quite appreciable. In mineral media with continuous aeration, the values of  $k$  tended to be proportional to light intensity at low light values, but finally approached a limiting value of 0.47 in the highest light intensity used and at 24.5° C. This maximum growth rate was the same with or without glucose and it strikingly resembles that of *Chlorella* at 23° C.

The nature of these results suggests very definitely that increased light is effective mainly by producing additional effects in the post-exponential phases of growth. The negligible effects of increases in light intensity during exponential growth seem to be explained most easily by supposing that even weak light produces some alteration in protoplasmic state which permits all



metabolic reactions to proceed at a much higher rate. It seems clear from the negligible effects of further increases in light intensity (Fig. 2) that photosynthesis is unimportant in this stage, presumably because in the presence of abundant sugar, photosynthesis is either unnecessary or suppressed. It

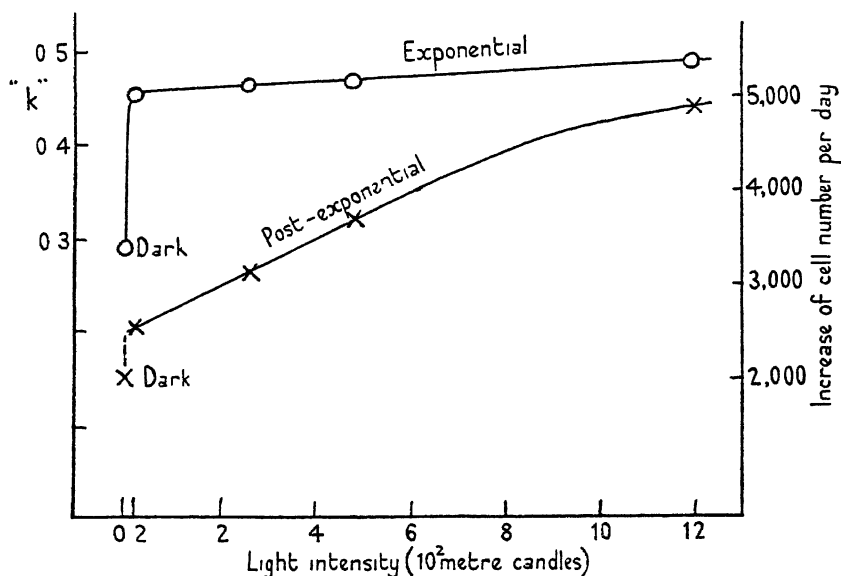


FIG. 2. Rates of growth at different light intensities. Upper graph, value of  $k$  during exponential period. Lower graph, the daily increase in cell number in the main post-exponential phase.

might, of course, also be possible for this process to be limited by low carbon dioxide supply to the small amount possible in the weakest light (series D). However, no increase in the exponential growth rate is produced in high light intensity (600–700 m.c.) by the addition of 2 c.c. of 8 per cent. potassium bicarbonate solution to the culture medium; so that there is no evidence that the growth process is limited by carbon dioxide shortage at this stage. Hence it is concluded that most probably light affects the protoplasmic state—this effect being evident in light of low intensity.

When exponential growth ceases, however, higher light intensities produce very marked increases in growth rate, as shown by the data in Table III.

TABLE III

*Effect of Light Intensity on Post-exponential Growth*

Series . . . . .	A.	B.	C.	D.
Light intensity ( $10^3$ m.c.) . .	12	4.8	2.6	0.2
Daily increase in number (days 8–14) and S.E.	$4880 \pm 138$	$3680 \pm 77$	$3150 \pm 68$	$2530 \pm 71$
Ratios of above	1.93	1.45	1.24	1.00
Rate factor $r$ (days 6–14)	1.95	1.47	1.25	1.00

The results are also expressed, graphically, in Fig. 2, which shows that the growth rate is at first proportionate to increased light intensity, but shows a falling off at 1200 m.c. (series A), the highest light intensity used. At this light intensity the growth rate appears to approach a limiting value, and it is clear from our other results that we cannot expect a greater average increase than 5,000 cells per day in this growth phase at 23° C. It is of interest to note that this limited growth rate is approximately twice that at the lowest light intensity.

An examination of the data shows that, up to light intensities of about 7-800 m.c., the growth rate/light intensity relation must be of the type:

$$\begin{aligned} r_1 &= r + rlt \\ &= r(1 + lt), \end{aligned}$$

where  $r_1$  is the number increase per day at a given light intensity  $I$  (in  $10^8$  m.c.),  $r$  is the number increase per day in weak light of negligible photosynthetic effect, and  $t$  is time in days. Thus we get:

Light intensity ( $10^8$ m.c.)	0.02	0.26	0.48	1.20
$(1 + lt)$ calculated	1.02	1.26	1.48	2.20
$(1 + lt)$ observed*	1.02	1.27	1.49	1.98

\* Adjusted from Table III.

It is assumed that this direct proportionality between growth rate and light intensity implies that at this stage light intensity affects the rate of photosynthesis and hence growth. It seems that a limiting factor other than light intensity reduces the growth rate at 1200 m.c. (A) and it is suggested that the limiting factor may be deficiency of carbon dioxide. None is supplied in the medium, and that available must therefore be only that going into solution from the air and that produced in respiration.

While photosynthesis is assumed to cause the differences in the growth rate at this stage, there is no evidence that it is effective through increasing sugar production or concentration. Although Pearsall and Loose (1937) found that the cessation of exponential growth was associated with a fall in the internal hexose concentration, no significant difference in this concentration has been found in comparing either dark with light cultures, or those from low and high light intensities (3 examples only), at comparable stages of growth. Hence it must be assumed for the present that the rates of hexose absorption, or formation, balance or exceed the rates of growth.

It is possible that the fall in hexose concentration found when exponential growth ceases might be due to a decrease in the oxygen supply to the cells, because it has been necessary to assume that oxygen deficiency tends to limit post-exponential growth in darkness (Pearsall and Bengry, 1940). Presumably this would reduce the rate of hexose absorption, just as it would affect the rate of growth. Thus it is worth considering the possibility that oxygen supply is a controlling factor in the various light intensities used in the present instance. It will be necessary to suppose that different light intensities are effective in producing oxygen at different rates.

A parallel and well-known case of the limitation of growth by oxygen supply was studied by H. Brown (1914), that of yeast grown in malt wort. In this case, after a period of exponential growth, there is a period of constant number increase per unit of time. This linear rate of increase can only be explained on the assumption that when cell number is doubled, the *oxygen charge per cell* is halved, so that the growth rate now falls to one-half and the generation time is doubled. This gives a linear graph for the number/time relation. The resemblance between the growth of yeast in malt wort and of *Chlorella* in light is quite striking, for exponential growth ceases in the former about 4,000 cells per c.mm. and in the latter at 5,000 cells. Yeast then grows in a linear fashion for two days and it is tempting to compare this with the first linear phase in *Chlorella*, also two days in duration. If this is the case, however, what is the explanation of the slower growth in the second linear phase?

There is another way in which oxygen limitation could be effective. When the cells settle to the bottom of the flask, they may become dependent upon oxygen diffusing through the supernatant liquid. In this case, only the uppermost layer or layers of cells could absorb sufficient oxygen through the liquid. In accordance with this, the second linear period of growth may, therefore, represent a stage at which the oxygen charge on the cells as a whole falls below the limit requisite for growth and is not raised to it during the daily shakings. Under these conditions only the upper layers of cells (in the film developed on standing) can acquire sufficient oxygen to grow. This idea can be confirmed in part by measuring the oxygen absorption of cultures with the Warburg manometric technique. In measurements, for which we are indebted to Dr. G. C. Whiting, the cell suspensions were allowed to stand overnight in the Warburg flask and the changes in pressure due to oxygen absorption measured. The depth of liquid was rather less than in the culture flasks. Under these conditions in darkness or weak light the average rate of oxygen absorption was constant and independent of the cell number, over a wide range, 20–50,000 cells per c.mm. (The oxygen absorption on standing is less than one-eighth of that on shaking—and of course the latter varies with the number of cells present.)

There is thus little doubt that the supply of oxygen must become limiting in the later stages of growth. Further, if this effect operates, then the effect of light intensity on growth is probably indirect through the production of oxygen in photosynthesis. The *maximum* rate of photosynthesis must be determined by the rate of production of carbon dioxide plus that absorbed in solution. Hence the maximum rate of growth, varying as the oxygen in solution or absorbed plus the oxygen formed in photosynthesis, should be of the order of twice that observed in light too weak to give appreciable photosynthesis. In other words, the oxygen absorbed in the very weak light will appear as carbon dioxide which will be wholly converted to oxygen in strong light. Thus the fact that the maximum observed growth rate in light is of the

order of twice that in weak light seems to be a not unimportant corollary of the oxygen hypothesis. On the whole, then, the hypothesis that the effects of light intensity upon the post-exponential growth operate through the photosynthetic production of oxygen, is one which agrees well with the available facts and is worth detailed consideration.

The comparison of these growth rates in the light with those in darkness is much less simple, because of the difficulty of being certain which parts of the growth curves are equivalent. The corresponding 'dark' curve at 23° C. is taken from an earlier paper (Pearsall and Bengry, 1940) and the problem really depends on whether the point of inflexion at day 15 in the 'dark' curve (see Fig. 1) is equivalent to those at day 8 or to those at day 14 in the 'light' curves. If the exponential period lasts six days in light it should last about nine in darkness. Thus the first linear phase should start on day 9 and last six days in darkness. The rates of growth in this phase are then of a similar order of magnitude in weak light (2,530 cells per day) and darkness (2,000)—an expected result if photosynthesis is very small in the former, for both should depend on a similar oxygen supply.

If, however, the point of inflexion on day 15 of the dark curve is equivalent to that on day 8 (light) as it is *in number*, then we must conclude that a small amount of light increases the growth rate (post-exponentially) by about three times. If we adopt this interpretation, we have to assume that a similar oxygen consumption (in darkness or weak light) produces three times as much growth in the latter, and we are also faced with the necessity of explaining why post-exponential growth is affected more by weak light than is exponential growth. In the latter the growth rate in weak light is only some 60 per cent. greater than that in darkness. Clearly the difficulties of explaining these features are very great, and without further evidence of their reality it is unlikely that this alternative treatment of the growth curves is correct.

Thus in accepting the first interpretation we assume that darkness has a small effect on the second linear phase though it greatly modifies the first linear phase.

The modification of growth by light in the first post-exponential phase requires further consideration in terms of the supposed effects of oxygen. Attention has already been drawn (p. 489) to the fact that up to day 4 very little change in exponential growth is discernible in light, although in the subsequent two to three days the differences become very pronounced. We interpret this as meaning that up to a cell number of about 1,000 or 1,500 per c.mm. no oxygen deficiency tends to appear. However, the cultures in light, and especially those in higher light intensity, will be able to produce oxygen and so will go on growing exponentially for a longer time and to a larger number. Thus they will finally enter the stage (phase 2) when oxygen charge determines the rate of growth, with a larger number of cells bearing the full oxygen charge. Their growth in the first post-exponential period will therefore depend on this continuation of exponential growth as well as upon

a smaller but continuous effect of the rate of oxygen production in light. Thus, the actual rates of growth will be numerically much higher in higher light intensities, although logarithmically they should be of the same order of magnitude as in the lower, after the initial differentiating effect of oxygen has disappeared. In the data available, the time intervals of one day are too long to allow an accurate examination of this point, but a comparison of the figures for series A and D, day 6 to 8, suggests that it is essentially sound.

Thus the oxygen hypothesis is adequate to account for all the more important features of the growth curves, at least qualitatively if not always quantitatively. Its adequacy or otherwise does not, however, alter the essential conclusions drawn in this study, which are, that light intensity has comparatively little effect upon exponential growth in *Chlorella*, but a much larger effect upon post-exponential growth under the conditions used. On the other hand, the differences in growth rate caused by light in contrast to darkness are large during exponential growth and small after this has ceased. The differentiating effects of light or of light intensity on the growth curves are very marked about the time when exponential growth ceases.

#### SUMMARY

1. The alga *Chlorella* has been grown in glucose media in light of different intensities.
2. Increasing the light intensity has comparatively little effect upon the rate of growth in the exponential stage—although a large effect is produced by the weakest light employed (10 m.c.) when compared with darkness.
3. Light intensity markedly affects the rate of growth in the post-exponential stages.
4. The post-exponential growth curves tend to take up linear and standard forms, with at least two phases.
5. Oxygen is assumed to be limiting growth in these stages, at first owing to the decreasing oxygen charge per cell, and subsequently owing to the limited diffusion of oxygen into the medium.
6. Various features of the growth curves can be explained in the light of this hypothesis.

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# The Interaction of Factors in the Growth of Lemna

## XV. On a Rhythmic Growth Cycle of Lemna Colonies associated with Transference to a Potassium-free Nutrient Solution

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With four Figures in the Text

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### INTRODUCTION

THE effects of potassium on metabolism, that have been discussed in previous communications of this series (White, 1936, 1938*a*, 1939), have been based on an analysis of the data of growth of *colonies*, and attention was not directed in these communications to other effects, which require for their interpretation consideration of the growth of *single* plants and fronds.

### EXPERIMENTAL RESULTS

It has been established in previous communications (White, 1936, 1939) that potassium deficiency is associated with high dry weight per unit area. The dry weight per unit area of samples of colonies transferred from conditions of full nutrient supply to potassium-free nutrient solution (continuous illumination of 450 ft.-candles at 25° C.) in comparison with the dry weight of colonies transferred to nutrient solution with a concentration of 1 mg. potassium per litre is shown in Fig. 1 (see also White, 1936, Table V), a marked rhythmic fluctuation being apparent in the case of the potassium-starved colonies. Two strains of Lemna, having different growth-rates under similar conditions, were used in this experiment. During the first part of the experiment sampling was carried out from one strain and during the second part from the other, the difference in metabolism being reflected in difference in level of dry weight per unit area associated with potassium starvation. On each sampling occasion duplicate samples were taken and from the agreement between these duplicates the standard error of the

mean of two random samplings of the potassium-starved colonies has been calculated to be 3.2 per cent., whereas the order of fluctuation in dry weight per unit area between successive samples taken at approximate intervals of two days is 15 per cent. The amplitude of the periodical fluctuation in level of dry weight per unit area of the potassium-starved colonies is, therefore, of

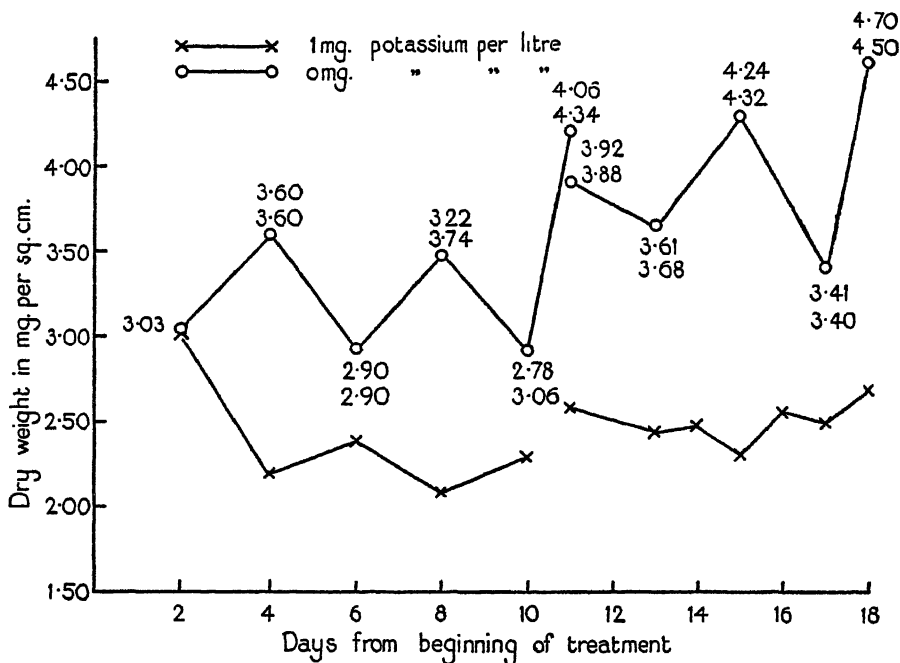


FIG. 1. Dry weight per unit area of colonies transferred from conditions of full nutrient supply to (a) potassium-free nutrient solution, (b) solution with potassium concentration of 1 mg. per litre. Two strains with different growth rates were allotted to each treatment, the break in the curves on the 11th day reflecting change in sampling from one strain to the other. Duplicate values of the potassium-starved colonies are inserted for comparison with the range of periodic fluctuation.

such magnitude as to preclude the possibility of its being a chance effect of random sampling. There is an indication of a periodical fluctuation in the dry weight per unit area of the colonies grown with 1 mg. potassium per litre, although not sufficiently marked to be considered definite, whereas in colonies with 300 mg. potassium per litre (White, 1936, Table V) there is no indication at all of rhythmic fluctuation. It appears, therefore, that the periodicity of growth is associated with the omission of potassium from the nutrient solution.

The general rate of increase in frond number of potassium-starved colonies has been shown to conform with the differential equation  $\frac{1}{n} \frac{dn}{dt} = \frac{r}{t}$ , where  $n$  represents frond number,  $t$  the time elapsed since the omission of potassium

from the nutrient solution and  $r$  is a constant (White, 1936, p. 178). Whereas sampling to provide fronds for the area and dry-weight estimations plotted in Fig. 1 was carried out on every second day the numbers of fronds in the colonies were counted every day, allowance being made in calculating the growth rate of the colonies for those removed in the taking of samples.

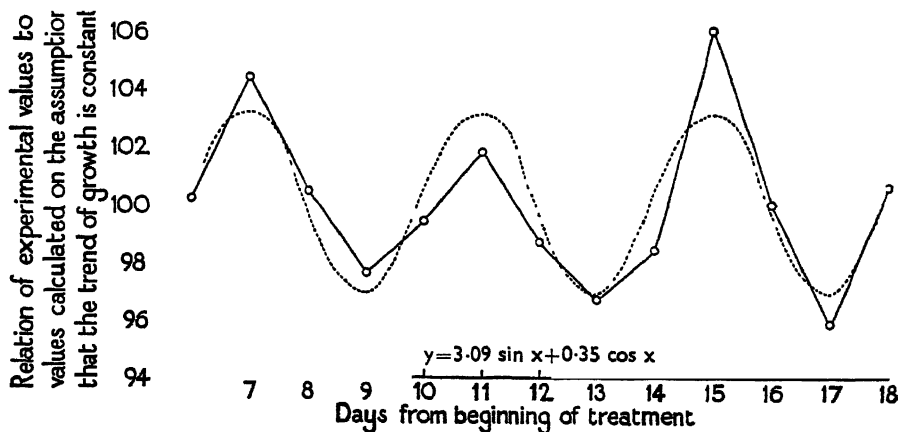


FIG. 2. Deviation of frond numbers of a potassium-starved colony from values calculated in accordance with the differential equation  $\frac{1}{n} \frac{dn}{dt} = \frac{r}{t}$ . ( $n$  = frond number,  $t$  = time, and  $r$  = constant), which represents the general drift in growth rate of the colony with time. This trend in growth rate is calculated from one day after transference of the colony to a potassium-free solution ( $r = 0.710$ ).

These frond counts are available for the first part of the experiment only in the case of the colony sampled during this period, which was discontinued after the eleventh day, but over the whole period of the experiment in the case of the colony that was sampled from the eleventh to the eighteenth day. The frond numbers of the latter colony are plotted in Fig. 2 on a relative basis, the general drift in time being eliminated by using the equation referred to above and each of the values plotted in the diagram being stated as a percentage of the corresponding smoothed value. Three complete cycles of growth are apparent covering the period from the sixth to the eighteenth day of the experiment, and the harmonic curve of closest fit to the data has been inserted in the graph.

Whereas the rhythmic cycles of Fig. 2 extend from the sixth to the eighteenth day of the experiment, sampling only began on the eleventh day. Prior to this period fronds were not removed for area and weight estimations and the colony was allowed to increase undisturbed. It is difficult, therefore, to attribute the cyclic effect that we are here concerned with to the indirect result of sampling, recently claimed by Dickson (1938 *a*) to be the cause of rhythmic growth cycles of *Lemna* colonies. Indeed, it is evident from Fig. 2 that the introduction of sampling from the eleventh day of the



experiment onwards has had no apparent effect on the amplitude or frequency of the cycles.

#### PREVIOUS RECORDS OF RHYTHMIC GROWTH CYCLES OF LEMNA COLONIES

Before discussing the case of rhythmic growth illustrated in Figs. 1 and 2, previous work on the causation of rhythmic growth cycles of *Lemna* colonies must be referred to. Ashby, Bolas, and Henderson (1928), in the first communication of this series, stated that a rhythmic fluctuation occurred in their growth curves of plant number. In early experiments the present writer encountered cases of rhythmic growth, but on further investigation such cases were invariably found to be accounted for by inadequate control of the experimental conditions. Thus a definite and clear-cut rhythm in growth rate of colonies, which were counted every day and transferred to fresh nutrient solution every second day, was completely eliminated when care was taken to adjust the temperature of the fresh nutrient solution to that of the growth chambers prior to transference of the plant colonies. The occurrence of rising or falling trends of growth persisting over several months, even under constant conditions, was, however, demonstrated (White, 1936 *a*), and it was tentatively suggested that these trends corresponded with the inherent annual cycle of growth rate under natural conditions, that had not been entirely eliminated by transference of the colony to a controlled environment. Dickson (1938, 1938 *a*) claims to have demonstrated rhythmic growth cycles of *Lemna* colonies of both long (25–40 days) and short (4–6 days) duration and asserts that these rhythmic growth cycles arise from and are maintained by the process of sampling.

There is no need to discuss here the experimental evidence for Dickson's long-wave fluctuations, since those shown in this experiment are of short duration. Neither is it intended here to discuss the experimental evidence that Dickson puts forward to support his views concerning the short-wave fluctuations. He undoubtedly shows the occurrence of short-wave fluctuations in his experiments, but the view does not carry conviction that these fluctuations are due entirely to the sampling of his colonies. It is necessary, however, to point out that his theoretical treatment of the problem is inadequate. Dickson (1938 *a*, Table I) introduces a theoretical demonstration involving the assumption that daughter fronds are produced at four-day intervals and therefore that one-quarter of the original number of fronds will 'divide' every day. The theoretical values taken from Dickson's paper are reproduced as part of Table I, in which are shown the effects of daily reduction by sampling on the estimated factor of increase. Dickson concludes that the variation in this factor of increase is due to the reduction by sampling. In the same table, however, a similar estimate is made without sampling, using the same assumptions, and it is seen that the factor of increase varies in exactly the same way. It would appear, therefore, that Dickson is incorrect in attributing the variation in the factor of increase to reduction of numbers

by sampling. Indeed, the variation in his factor of increase would appear to be dependent on the inherent assumption that the fronds 'divide' or double in number at specified intervals like the units of a colony of bacteria. This assumption is clearly incorrect, and the actual mode of increase of a Lemna colony is re-examined in a later section of this communication. It may be concluded here that, in general, rhythmic growth cycles are not necessarily at all related to sampling methods.

TABLE I

*The Rate of Increase of 400 Fronds on the Assumption (following Dickson, 1938 a) that the Time from the First Appearance of a Frond to that of its First Daughter is Four Days and that One Quarter of the Original Number of Fronds Double in Number each Day.*

(a) With Sampling								
Original fronds.	Count 1st day.	Reduction to 400.	Count 2nd day.	Reduction to 400.	Count 3rd day.	Reduction to 400.	Count 4th day.	Reduction to 400.
100	200	160	160	133·3	133·3	114·5	114·5	100
100	100	80	160	133·3	133·3	114·5	114·5	100
100	100	80	80	66·6	133·3	114·5	114·5	100
100	100	80	80	66·6	66·6	57·3	114·5	100
Total frond numbers and factors of increase.		$\frac{500}{400} = 1·25$	$\frac{480}{400} = 1·20$		$\frac{466·6}{400} = 1·17$		$\frac{458·0}{400} = 1·14$	

(b) Without Sampling				
Original fronds.	Count 1st day.	Count 2nd day.	Count 3rd day.	Count 4th day.
100	200	200	200	200
100	100	200	200	200
100	100	100	200	200
100	100	100	100	200
Total frond numbers and factor of increase.		$\frac{500}{400} = 1·25$	$\frac{600}{500} = 1·20$	$\frac{700}{600} = 1·17$
				$\frac{800}{700} = 1·14$

### THE GROWTH CURVE OF A SINGLE FROND

The unit of growth in Lemna is known as a 'frond', a term that evades the problem of whether the structure referred to is a leaf or foliose stem. In order to determine the growth curve of a single frond the development of a colony from a single plant was followed in detail. Each plant, after natural separation from its neighbour, was transferred to a separate tube of nutrient solution and all fronds were numbered in their order of production. By the method described by Ashby, Bolas, and Henderson (1928) the outlines of the plants constituting this clone were traced daily for twenty-one days, and on these tracings the outlines of the single fronds were inserted at the time so

that the areas of individuals could afterwards be determined with a planimeter. From the permanent record thus obtained the growth curves of single fronds have been prepared, and a typical curve is illustrated in Fig. 3.<sup>1</sup> Attention is directed by arrows to the times of first appearance of daughter

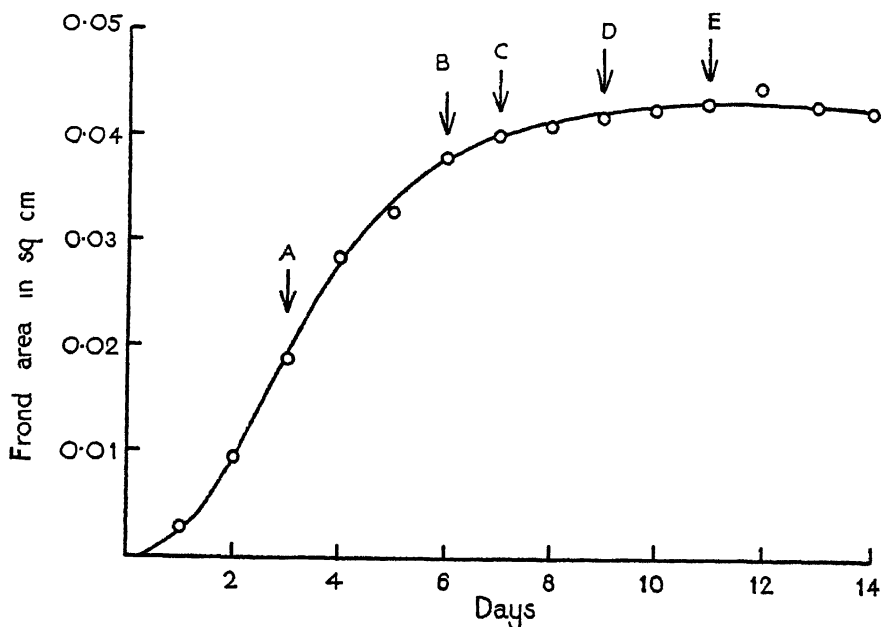


FIG. 3. Growth in area of a single Lemna frond. At the arrow marked A the frond attained sufficient size to be reckoned as a separate unit in the counting of the colony. At the arrow marked B the first daughter frond became visible. At the arrow marked C this frond attained sufficient size to be reckoned as a separate unit. At the arrow marked D the second daughter frond became visible. At the arrow marked E this frond attained sufficient size to be reckoned as a separate unit.

fronds and at which these fronds had grown sufficiently to be reckoned as separate units. It is to be noted that the growth curve is uniformly smooth nor is any irregularity or rhythm of growth caused by the appearance of daughter fronds, which develop before the parent frond has reached full size.

#### THE MODE OF INCREASE OF A LEMNA COLONY

The units composing a Lemna colony are plants which consist under the conditions of this series of experiments of two or more fronds. Each Lemna

<sup>1</sup> The area of an immature Lemna frond, as experimentally determined, is not the true area, for the fronds are produced in pouches on each side of the axis of the parent frond and during the early stages of growth much of the frond is hidden. Occasionally a frond ceases to grow and to reproduce and is thrown off from the parent frond without any daughter fronds upon its margin. Such a frond was measured and the areas (sq. cm.) obtained at daily intervals were 0.0029, 0.0138, 0.0246, 0.0246 (here the frond separated from its parent), 0.0398, 0.0384, 0.0398. This suggests that as much as 62 per cent. of the area of a frond may be concealed by the parent.

frond bears a daughter frond in a pouch on either side of the parent frond. Subsequently these daughter fronds, each of which is already producing its own daughter fronds when emerging from the pouch, separate and the whole process is then repeated. The writer has traced the production of four

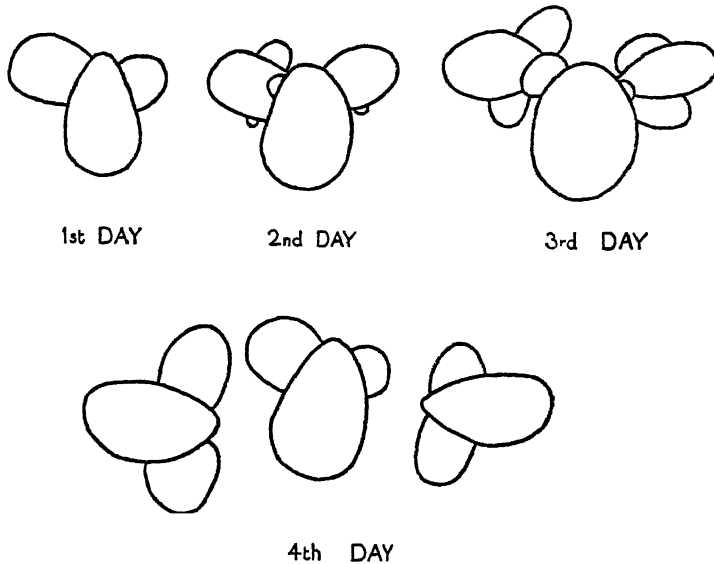


FIG. 4. The mode of increase in number of fronds of a Lemna colony. The growth cycle illustrated is that of a colony under continuous light of 1,200 ft.-candles at 25 C.

generations of fronds on each side of a parent frond, while Winter (1937) shows that as many as six are produced during the life of the parent frond.

In order to investigate the bearing on the growth curve of the whole colony of the production of fronds in this way a number of plants, selected so as to be all in the same stage of development, were isolated and their progeny followed through several generations. Fig. 4 shows the mode of production of new fronds from a single parent plant during one complete growth cycle, which, under the continuous light intensity of 1,200 ft.-candles here used, required a period of three days. The experiment was repeated under four different light intensities, and at each light intensity was duplicated with substantially similar results, although at lower light intensities a longer period was required to complete the cycle. The number of fronds to be counted each day are 3, 4, 7, and 9, which correspond with relative growth rates (differences between natural logarithms) of 0.288, 0.551, and 0.251. This suggests that a colony developed under controlled conditions from a single plant would be characterized by a periodicity in the rate of increase in frond number. This consideration presupposes, however, that the fronds produced in serial order from a given parent frond are developed at a uniform rate. Winter (1937) has shown that this rate is not constant but lengthens

as the parent frond ages. Presumably under these circumstances the rhythmic changes in frond numbers that would otherwise be maintained in a colony grown from a single plant would disappear through simultaneous development of fronds with different growth rates. Confirmation of this view is afforded by the absence of any indication of rhythmic rate of increase in frond number in the authors' experiments in such established colonies as have been derived from a single plant.

#### DISCUSSION

Although the rate of increase in frond number of an established colony does not normally show a rhythmic cycle, it is evident that the *individual* plants composing the colony must each be passing through such a cycle of growth as is illustrated in Fig. 4. If at any time any factor were to throw a major proportion of the plants in the colony into one phase of this cycle, then this would be reflected in a rhythmic growth cycle of the colony as a whole. The frequency of such a cycle would correspond with the period between successive frond generations. It may be noted that the rhythmic growth cycles illustrated in Figs. 1 and 2 are associated with a high residual potassium concentration in the fronds on the initial day of starvation, for maximal growth under the experimental conditions was obtained with a concentration of 20 mg. per litre (White, 1936, Table III), whereas the colony had been growing prior to the starvation period in the supra-optimal concentration of 300 mg. per litre. The first effect of increase in frond number subsequent to transference of the colony to potassium-free solution should therefore be a reduction of the residual potassium concentration to levels compatible with an increase in growth. This is confirmed by the experimental results, for during the first two days after transference of the colony to a potassium-free solution the growth rate rose to a level 34 per cent. higher than that calculated on the basis of the rate of falling-off during the subsequent period of starvation and 12 per cent. *higher* than the growth rate of a sub-colony continued with full nutrient supply. It appears that a relatively large number of fronds were added to the colony in a short space of time and these presumably tended to develop simultaneously and to produce daughter fronds simultaneously, thus giving rise to a rhythmic growth cycle.

Since at any time each frond in a *Lemna* colony is bearing neither more nor less than two daughter fronds the duration of the period in days between successive frond generations may be obtained from the formula  $1.0896/r$ , where  $r$  is the relative growth rate (daily difference between natural logarithms of frond number). If  $r$  is 0.290, the experimentally determined growth rate of a colony with full nutrient supply but in other respects under conditions identical with the potassium-starved colony of Fig. 2, then the duration of the period between successive frond generations is estimated to be 3.8 days, a value in close agreement with the frequency of four days used in Fig. 2 in

fitting a harmonic curve to the frond numbers of the potassium-starved colony. It appears that a proportion of the fronds of the potassium-starved colony are maintaining the same period between successive frond generations during potassium starvation as with full nutrient supply, although this is obviously not true of the whole colony, for the mean growth rate is falling throughout the starvation period.

Reference may here be made to a well established feature of potassium starvation in other plants—the localization in a potassium-starved plant of the residual potassium in the terminal bud (Nightingale et al., 1928; Janssen and Bartholomew, 1929). The result of this localization is that in barley (Gregory, 1937) and tomato (White, 1938) the development of the terminal bud of a potassium-starved plant is maintained at the same rate as, or even slightly in excess of, that of a completely manured plant, despite the falling off in growth rate of the plant as a whole. As described by Blodgett (1915), the first daughter frond formed from a parent frond has in *Lemna* the character of a terminal bud, while subsequent daughter fronds are axillary in position and may be considered to originate as axillary buds. Since any growth subsequent to the transference of a *Lemna* colony to a potassium-free solution must be attributed to the residual potassium in the fronds on the initial day of starvation, it is evident that continual translocation of this residual potassium to successive terminal buds (first-formed daughter fronds) would result in the maintenance of the period between the production of a frond and its first-formed daughter. The supply of potassium in a starved plant is, however, necessarily limited and the rate of production of subsequent fronds would therefore be retarded, leading to falling off in mean growth rate of the whole colony. The association of rhythmic growth with potassium starvation is compatible with the view that in a *Lemna* colony the supply of residual potassium in each plant under conditions of starvation is mobilized at the primary growing point, which is thus able to maintain the same rate of development during starvation as with full nutrient supply.

#### SUMMARY

The mode of production of fronds by a *Lemna* plant and the growth curve of a single frond are discussed. It is shown that rhythmic growth cycles do not necessarily arise through the process of periodical removal of fronds for sampling, as previously claimed by Dickson (1938 *a*).

A rhythmic growth cycle in dry weight and frond number of a potassium-starved colony is demonstrated. A temporary rise in growth rate immediately following transference of the colony to a potassium-free solution and the agreement between the duration of the cycles and the period between frond generations of a colony growing under similar experimental conditions, but with full nutrient supply, lead to the view that this rhythmic cycle is (*a*) set up through the sudden transference of a colony with supra-optimal potassium supply to a potassium-free solution, resulting in a sudden temporary increase

in frond production, and (b) maintained by translocation of the residual potassium in the fronds on the initial day of starvation through successive generations of first-formed daughter fronds, which develop at the same rate with potassium starvation as with full nutrient supply.

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# Injection for the Diagnosis of Mineral Deficiencies in the Tomato, the Potato, and the Broad Bean<sup>1</sup>

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With Plates II to IV and six Figures in the Text

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<sup>1</sup> This article is part of a thesis submitted by one of us (H. H.) for the degree of Doctor of Philosophy in the University of London and describes work carried out at the East Malling Research Station, Kent.



## INTRODUCTION

IN a recent paper Roach (1938, 1939) described how certain methods of injection can be used for diagnosing mineral deficiencies. He pointed out that the methods have to be adapted in varying degrees to suit each plant. The present paper describes methods proved suitable for the tomato, the potato, and the broad bean and gives proof that the methods are satisfactory for diagnosing deficiencies of nitrogen, phosphorus, potassium, calcium, magnesium, and boron.

The movement of injected liquid varies markedly from species to species, and it is found convenient to carry out preliminary experiments with dyes the movement of which can be watched, often without even damaging the plant, to decide rapidly which injection methods are worth testing finally with actual nutrient solutions. For each plant the results of experiments with dyes was followed by others in which the movement of injected nutrient solutions was ascertained by the increase in growth or improvement in leaf colour. For this purpose plants were grown in sand supplied with controlled nutrient solutions.

*Nutrient solutions.*

The following nutrient solutions were made as described by Hill, Davis, and Johnson (1934) except that for the potato these factors have to be applied to the amounts supplied: nitrogen 1.5, phosphorus 1.47, potassium 2.23, and calcium 0.25. (i) Complete nutrient solution less nitrogen. (ii) Complete nutrient solution less phosphorus. (iii) Complete nutrient solution less potassium. (iv) Complete nutrient solution less calcium. (v) Complete nutrient solution less magnesium. (vi) Complete nutrient solution less boron. (vii) Starved of all the above elements.

## THE TOMATO

1. *Cultural notes.*

Ordinary clay pots were cleaned, heated, and dipped into almost boiling paraffin wax until impregnated. The drainage hole was covered with a pad of glass wool to retain the fine Bedfordshire silver sand (99 per cent. insoluble in hydrochloric acid) employed. The seed was germinated in this sand and when the leaves were well developed the plants were pricked out into 2-in. pots. When the plants had reached a height of 4–5 in. they were finally transplanted into 4-in. clay pots filled with the same sand.

The first 'feeding' was given two or three days after the pricking out into 2-in. pots, and thereafter at intervals of a week. The plants were fed at first at the rate of 50 c.c. of nutrient solution per pot, which was increased to 200 c.c. per pot when transplanted into 4-in. pots. Only one-fifth the full concentration of the nutrient solution was employed for the first supply,

subsequent applications increasing by a fifth until the full concentration was reached.

## 2. *Deficiency symptoms.*

(i) *Nitrogen.* Nitrogen-starved plants were characterized by slow, slender growth with thin, light green to yellow-green leaves, the symptoms commencing on the lower leaves but progressing rapidly upwards until the entire plant was affected. There was a progressive dying and dropping of the older leaves.

(ii) *Phosphorus.* At the time of injection the leaves were small and of a dull purplish-green colour. The veins and undersides of the leaves were decidedly reddish-purple.

(iii) *Potassium.* Conspicuous symptoms of the lack of potassium were relatively slow to appear compared with those of phosphorus or nitrogen. Incipient potassium deficiency in the presence of the ample supply of nitrogen was first noticeable in the leaf by the occurrence of a very dark green or slightly bluish-green colour. Later the lower margins of the leaves became chlorotic, ultimately becoming scorched until finally the whole leaf turned greyish-green and dried up. There was a gradual upward progression of these symptoms, until only the young leaves at the growing tip were unaffected by marginal chlorosis or scorching.

(iv) *Calcium.* The effect of calcium deficiency on the foliage was unlike that of any of the deficiency symptoms previously described, in that the young upper leaves were first affected. These became decidedly yellow, the tissue bordering the midrib remaining green the longest. The terminal bud died; the lower leaves were somewhat thickened, but normal in colour.

(v) *Magnesium.* The effect of magnesium deficiency was somewhat similar to that of potassium in that the initial symptoms consisted of a marginal chlorosis and scorching of the lower leaves, the symptoms progressing upwards. In magnesium deficiency the chlorotic areas were white, with a dark brown or black line bordering the green unaffected tissue, while in potassium deficiency the chlorotic areas were yellowish-brown.

(vi) *Boron.* Boron deficiency symptoms were first observed on the extreme lower leaves which became a lighter green and later developed typical orange blotches between the veins which progressed outwards from the midrib. Symptoms progressed slowly upwards, the lower leaves gradually dying and falling. The upper immature leaves remained green and healthy in appearance. The stems and petioles became weak and brittle.

(vii) *All nutrients.* Plants which received no nutrients soon exhibited the same symptoms as those with phosphorus deficiency. These symptoms masked any others which might otherwise have been observed.

## 3. *Interveinal leaf injections.*

(a) *Experiments with dyes.* Plate II, Figs. 1, 2, 3, shows the distribution resulting from the injection of 0.02 per cent. solution of acid fuchsin into

leaflets of increasing sizes and ages through incisions (indicated by arrows) made in interveinal areas, by the method described by Roach (1938, p. 21). Injection was allowed to proceed until the capillary tube container was emptied (usually in 2–3 hours). It will be seen that in the two smaller leaflets there was some movement across the secondary veins; whereas in the largest leaflet the better developed secondary veins acted as efficient barriers to the further distribution of the dye.

This type of injection, therefore, is not likely to give, with the tomato, the clear-cut effects obtained with the apple (Roach, 1938, p. 18).

(b) *Experiments with nutrients.* (i) Numerous injections were made of leaflets in various stages of physiological maturity with solutions of 0.5 per cent. urea and 0.125 per cent. ammonium nitrate. There was no injury, but no definite beneficial result was observed. In a few immature leaflets there appeared to be a slight intensification of chlorophyll near the incision after a period of eight days, but not sufficiently marked to be of definite value for diagnostic purposes.

(ii) Interveinal injections were made (with 0.25 per cent. solution of disodium hydrogen phosphate) in immature leaflets. In 8–10 days a marked response was observed, characterized by the disappearance of the purple hue, and the appearance of a more normal green colour. The area of the leaflet affected corresponded closely to that permeated by the dye (Pl. II, Figs. 1, 2, 3). When injections were made before the deficiency symptoms had become so pronounced, that is, when leaflets were dull green in colour but before the purple colour appeared, diagnosis was not so definite (see also p. 509, para. vii).

(iii) *Potassium.* Interveinal injections were made with 0.25 per cent. and 0.5 per cent. potassium sulphate solutions of nearly mature leaflets exhibiting conspicuous deficiency symptoms and of immature leaflets only slightly abnormal green in colour. These injections produced no visible effect. Young leaflets not exhibiting deficiency symptoms were injected to determine whether the onset of symptoms might be retarded. The upward progression of deficiency symptoms was carefully noted, but no such effect was observed. Janssen and Bartholomew (1929) have pointed out that in the tomato the potassium is translocated from one region of the plant to another as needed, so that there is a progressive dying of the older leaves and the continued formation of new ones. For this reason, the injection of a leaflet not exhibiting deficiency symptoms may not prevent them from appearing, since the demand by the growing point may drain the element from the injected leaflet before its effect can be manifested at that point.

(iv) *Calcium.* Interveinal injections were made with 0.25 per cent. solution of calcium chloride or calcium acetate of young leaflets exhibiting distinct deficiency symptoms, but no response was observed. Possibly the deficiency was allowed to exert its influence too long before injections were made. The initial onset of symptoms was followed very rapidly by severe injury to the

meristematic tissues, thus bringing about a physiological maturity in very young leaflets.

(v) *Magnesium*. Interveneal injections were made with 0.25 per cent. solution of magnesium sulphate into leaflets in various stages of maturity, without securing any noticeable effect.

(vi) *Boron*. Preliminary injections with 0.1 per cent. boric acid solution produced definite injury in the form of flaccid greyish-green areas, which later turned brown and died. A 0.001 per cent. boric acid solution was employed for all later injections without causing any injury.

Injections were made of leaflets exhibiting symptoms in varying degrees of severity, but no effect was observed. It must be remembered that such leaflets are relatively mature. Injections were also made into less mature leaflets, not exhibiting deficiency symptoms, and in these definite retardation of the onset of symptoms was obtained, as shown in Plate II, Fig. 4. It will be observed that the effect is not restricted to the interveneal area injected, as with the dye (Figs. 1, 2, 3). The distribution of the boric acid was similar to but more widespread than that of the dye or of disodium hydrogen phosphate.

(vii) *All nutrients*. Injections of immature leaflets with a complete solution containing all six elements produced in ten days a beneficial effect similar to that noted with phosphorus-starved plants. As already mentioned, these plants have a striking resemblance to the phosphorus-deficient plants. The injection of 0.25 per cent. solution of disodium hydrogen phosphate brought about an improvement similar to that of the 'complete' nutrient.

#### 4. *Leaflet-tip injection.*

*Experiments with dyes*. When the tip of a lateral leaflet or not more than  $\frac{1}{16}$ th of its length was cut off and the cut end immersed in dye the leaflets on the same side of the midrib were permeated, while those on the other side were unaffected. Only the proximate half of the terminal leaflet was affected. Although the dye also travelled towards the base of the midrib, it did not reach the main stem after absorption had been allowed to proceed for twenty-four hours. If the tip of the terminal leaflet was thus injected the leaflets on both sides of the midrib were permeated and the dye did not reach the main stem after an absorption period of twenty-four hours. This offers a fairly sensitive method of comparing the effect of various substances injected into one plant, in the first case providing a comparison in the same leaf of injected and non-injected tissue separated by the midrib.

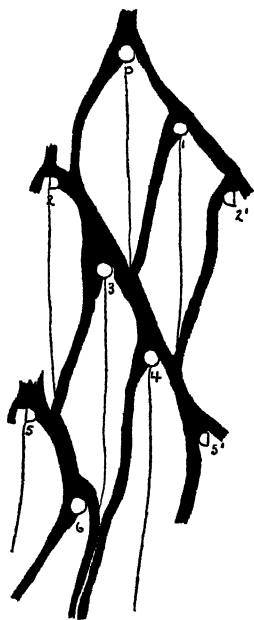
The author did not supply nutrient solutions through the leaflets, but Roach (1938 a) has employed it successfully on tomatoes growing in the open in the Cape Province of South Africa.

#### 5. *Leaf-stalk injection.*

(a) *Experiments with dyes*. In the leaflet-tip type of injection just described it was found that the farther the leaflet was cut back the more extensive

was the permeation. If injection through a cut leaf-stalk with the leaflets removed was allowed to proceed long enough all the foliage of the plant was affected, and if stopped earlier there was no marked line of demarcation between permeated and unaffected tissue. Distribution occurred more rapidly above the point of injection than below. Permeation of the entire foliage of a plant 24 in. in height took place in three hours on a fine bright day with the soil rather dry, when the leaf-stalk was cut midway and bent to dip into light green dye.

The movement of the injected dye in the plant was traced by peeling off the cortex and exposing the vascular strands (Text-fig. 1).



TEXT-FIG. 1. Distribution of organic dye in a tomato stem when injected by a cut leaf-stalk or adventitious root.

The leaves of the tomato plant are arranged spirally on the stem, their angular divergence being one-third. In Text-fig. 1 the stem is represented as split longitudinally and flattened out in order to show the relationship of the different leaf traces as shown by the coloured strands injected with dye. It will be seen that the leaf traces divide above each leaf base (numbered) joining up with the strands from the two leaves having an angular divergence of one-third, and also with the leaf directly below it. It is evident that there is a practically continuous vascular connexion between all leaf traces.

The leaf-stalk method therefore seems to offer a simple method of injecting the whole plant; and this can be repeated at intervals because the basal leaves are normally removed. Possibly these conclusions may have to be modified somewhat with tomato plants growing in the open.

(b) *Experiments with nutrients.* (i) *Nitrogen.* In the first instance, periodic injections were made of plants exhibiting moderate symptoms of nitrogen deficiency to supply a small amount of the lacking element, different compounds being used to ascertain the best kind and the concentration which would bring about a visible response without causing injury. 0.25 per cent. solution of urea or ammonium nitrate were found to be suitable. Plate III shows the effect of three injections of 0.25 per cent. solution urea (Fig. 10) compared with an untreated plant (Fig. 11). A marked response in growth and colour may be seen. A distinct intensification of chlorophyll took place in the young leaves in twelve days, although the greatest response was noted in the immature leaves and in a renewal of growth; the effect was general rather than localized. Owing to the small amount of foliage and high humidity in the greenhouse, only a few c.c. of solution were absorbed at each injection even when absorption was allowed to proceed for thirty-six hours. After this period, browning

and dying of the injected leaf-stalk occurred, and no further absorption occurred unless the petiole was cut back well beyond the injury.

A plant which had reached a fairly advanced stage of nitrogen deficiency was injected by cutting a leaflet half way and immersing the cut end in a solution of 0.25 per cent. ammonium nitrate for twenty-four hours. In fifteen days the young immature leaves had attained a healthier darker green colour. The plant was reinjected and after a further twelve days had made considerable healthy new growth (Pl. III, Fig. 12), while the check plant was about to die (Pl. III, Fig. 13).

(ii) *Phosphorus*. Plants exhibiting marked phosphorus deficiency symptoms, shown by stunted growth, and dull green leaves with purplish veins and under-surfaces, were injected with 0.25 per cent. solution of disodium hydrogen phosphate. Absorption was allowed to proceed for twenty-four hours. A distinct improvement in the colour of all the leaves was observed (Pl. III, Fig. 14) after 15–17 days in increased growth and the disappearance of purple coloration four weeks after two injections of 0.25 per cent. solution of disodium hydrogen phosphate. Plant 15 of Plate III was not injected.

(iii) *Potassium*. A plant was injected at intervals of three weeks with 0.25 per cent. solution of potassium sulphate. This provided the plants with sufficient potassium to make fairly vigorous growth and to prevent the occurrence of deficiency symptoms. This is shown in Plate IV, where a comparison is made between the injected plant 16 and another, 17, receiving the same nutrient solution but not injected. It will be observed that the injections have had a general, rather than a localized effect. In the untreated plant the marginal yellowing and scorching have reached a very advanced stage, affecting the plant up to the growing-point.

Plants which were already exhibiting marginal chlorosis and scorching of the lower leaves were also injected with 0.25 per cent. solution of potassium sulphate. After fifteen days a marked recovery was observed (Pl. IV, Fig. 18), indicated by a renewal of growth, the new growth being healthy in colour, and the arrest of the upward progress of the deficiency symptoms which proceeded in the untreated plants (Fig. 19). Subsequently the plant shown on the right was injected with 0.25 per cent. solution of potassium sulphate. After fifteen days the leaves at the growing-point had become healthier green in colour, and new leaves were unfolding. In eighteen days a marked recovery was apparent.

(iv) *Calcium*. Owing to the death of the terminal bud, quickly following the onset of symptoms, injections of this type were not attempted. There was no opportunity to test the effect of injection at an earlier stage of the disease.

(v) *Magnesium*. A plant receiving a magnesium-lacking solution was injected at intervals of three weeks with a 0.25 per cent. solution of magnesium sulphate. This provided the plant with sufficient magnesium to make fairly vigorous growth and prevent the occurrence of deficiency symptoms. When

plants were injected which were already showing deficiency symptoms the very young leaves at the growing tip became healthier green in colour and the progression of symptoms was checked.

(vi) *Boron*. Monthly injections into plants in the lacking-boron group were made with 0.001 per cent. solution of boric acid before deficiency symptoms had become apparent. Such plants grew vigorously and remained healthy and normal in all respects (Pl. IV, Fig. 20), whereas one (Fig. 21), receiving the same lacking-boron solution but not injected showed severe deficiency symptoms.

Plants exhibiting deficiency symptoms on the lower foliage were injected with 0.001 per cent. solution of boric acid. No effect was noticeable on the foliage already affected, but the upward progression of symptoms was checked and the plant made more vigorous growth.

## THE POTATO

### 1. *Cultural notes.*

Virus-free tubers (var. Majestic) kindly supplied by Dr. R. N. Salaman were sprouted in sand and allowed to root. The young plants were then separated from the parent tuber and transplanted into sand. Three plants were placed in each 7 in. paraffined clay pot and each pot was supplied with 400 c.c. of nutrient solution, once a week, the solutions being diluted to half their normal concentration for the first two applications.

### 2. *Deficiency symptoms.*

(i) *Nitrogen*. Potato plants deprived of nitrogen gradually lost their green colour, becoming yellowish-green and finally yellow; growth became slower and finally ceased.

(ii) *Phosphorus*. Plants deprived of phosphorus became stunted. The small dark green leaves arose at an acute angle to the main stem.

(iii) *Potassium*. Potassium deficiency showed itself first as a crinkling of the foliage which took on a dark bluish-green colour. Later the tips and margins of the leaves became chlorotic and brown patches appeared between the veins.

### 3. *Interveinal leaf injection.*

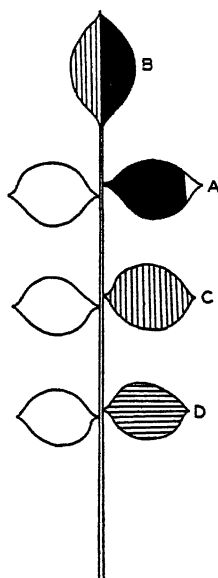
(a) *Experiments with dyes*. Plate II, Figs. 5, 6, 7, show the distribution resulting from the interveinal injection of 0.2 per cent. solution of acid fuchsin into leaflets of different sizes and ages. It will be seen that in none was the secondary vein crossed by the dye except close to the margin. The line of demarcation between permeated and untreated areas seemed sharp enough for this method to be used for diagnostic purposes. The difference between the potato and the tomato in this respect was most marked.

(b) *Experiments with nutrients*. The only nutrient injections were carried out on deficient plants when the foliage had attained a uniform light green

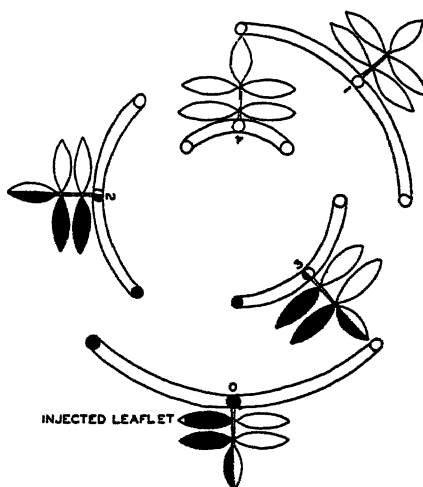
colour, leaflets of different ages and sizes being injected each with 0.25 per cent. ammonium nitrate, 0.5 per cent. urea, or 0.5 per cent. calcium nitrate. No beneficial effect was observed from any of these injections.

#### 4. *Leaflet-tip injection.*

(a) *Experiments with dyes.* When the tip of a lateral leaflet next to the terminal leaflet was cut off and the cut end dipped in dye, the nearer half



TEXT-FIG. 2. Leaflet-tip Injection of potato. Leaflet A injected: clear portion of leaflet cut off and the cut end immersed in dye. Solid black areas permeated in ninety minutes. Leaflet C permeated in three hours and a half; leaflet D and the farther side of leaflet B permeated at the end of twenty-four hours.



TEXT-FIG. 3. Basal leaflet-tip injection of potato: permeated areas black.

of the terminal leaflet, Text-fig. 2, was permeated in 90 mins. After absorption had proceeded for a further two hours the dye had passed down the midrib of the leaf and permeated the next leaflet C below on the same side of the midrib. When absorption was allowed to proceed for twenty-four hours, the dye had travelled to all the leaflets on the same side of the midrib as the injected leaflet. The leaflets on the other side of the midrib were unaffected, but both sides of the terminal leaf were permeated.

When one of the pair of leaflets nearest the main stem was thus injected the dye travelled into the next leaflet above, and backwards into the main stem, finally permeating the leaflets on the nearer side of the second and third leaves above the one injected. This is shown in Text-fig. 3, with the



leaves on a phyllotaxis diagram, in which is indicated the fact that the potato leaf is supplied by three vascular bundles, and that the leaf base subtends a quarter of the circumference of the main stem. The leaves are numbered up the stem, the injected one being 0. Thus the second and third leaves each have one-half of their leaflets permeated, and the other half untreated. Hence this method should prove useful for diagnostic purposes.

(b) *Experiments with nutrients. Nitrogen.* The tip of a basal lateral leaflet was cut off and the cut end dipped in 0.5 per cent. calcium nitrate. In eight days all the leaflets on the injected leaf and the leaflets on the nearer side of the second leaf above had become darker green. The third leaf above, which was only partially expanded at the time of injection, was also a healthy green colour on its nearer side. This result differed from that secured with dye injection in that the leaflets on both sides of the injected leaf were permeated. This slightly more extensive permeation of calcium nitrate than of dye is in harmony with the difference in their molecular size. However, in the second leaf above, there existed a clear-cut comparison between permeated and non-permeated tissue divided by the midrib.

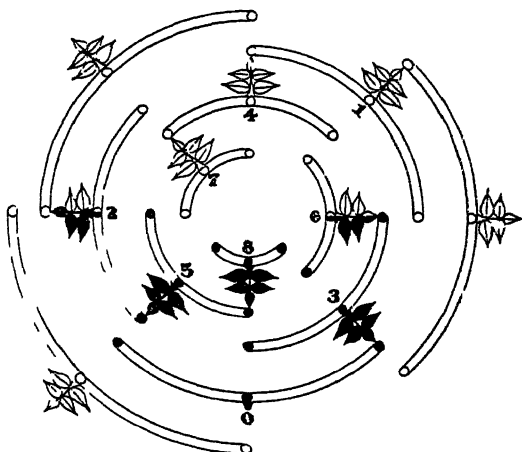
### 5. *Leaf-stalk injection.*

(a) *Experiments with dyes.* The leaflets were removed from a basal leaf, and one-half of the midrib was cut off. The cut end was then injected as described by Roach (1938). The extent of permeation could be noted in 2–3 hours. After absorption had proceeded for twenty-four hours there was no further distribution, but the colour was more intense.

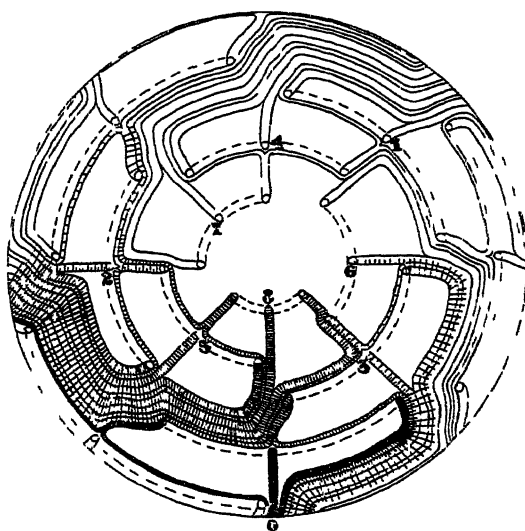
Angular distance from injected leaf.	No. of leaf above injected one.	Fraction of leaf injected.
3	1	None
2	2	Slightly more than half
1	3	Whole, but stronger on one side
0	4	None
1	5	Whole
2	6	Half
3	7	None
4	8	Whole

The distribution is indicated in the Table and is represented diagrammatically in Text-fig. 4, which is also drawn as if the leaves had been detached from the shoot and attached to points on a phyllotaxis diagram in the same manner as Text-fig. 3.

The leaves or fractions of leaves affected by the dye decreased as their angular distance from the injected leaf-stalk increased. Leaf 4 with an angular distance of  $\frac{1}{2}$ , and leaves 1 and 7 with an angular distance of  $\frac{3}{4}$ , were free of dye. Leaves 2 and 6 with an angular distance of  $\frac{1}{4}$  had one-half of



TEXT-FIG. 4. Phyllotaxis diagram representing the distribution of organic dye in the foliage of a potato shoot, when injected by a cut leaf-stalk.



TEXT-FIG. 5. Phyllotaxis diagram representing the distribution of dye in the conducting vessels of a stem of a potato shoot, when injected by a cut leaf-stalk.

their area permeated, while leaves 3 and 5 with an angular distance of  $\frac{1}{8}$ , and leaf 8 directly above the injected leaf, were entirely permeated.

The cause of such a distribution was revealed by stripping the cortex from the main stem and tracing the coloured leaf traces. This is shown in Text-fig. 5, in which the strands of the injected leaf 0 are represented by solid black

lines and the permeated strands are cross-hatched. Each leaf base is represented by a broken line and each leaf has three leaf traces. It will be seen that the leaf traces of leaves 1 and 7, having an angular distance of  $\frac{3}{8}$ , remain unpermeated. In leaf 2, with an angular distance of  $\frac{1}{8}$ , the right-hand and central trace are permeated, resulting in leaf permeation of slightly over one-half. In leaf 6, with the same angular distance, the left-hand and half the central trace are lightly permeated, while the right-hand trace is not affected, resulting therefore in permeation of only the left side of the leaf. The three leaf traces of leaves 3 and 5, with an angular distance of  $\frac{1}{8}$ , are all permeated, those of leaf 5 being permeated more heavily. The leaf traces of leaf 8, directly above the injected leaf-stalk, are all heavily permeated. The same injection pattern was obtained if the injected stalk was chosen so that leaves 6 and 7 were but partially expanded.

Obviously the most useful feature of the leaf-stalk injection method from the present point of view is the permeation of the second leaf above the injection point on one side of its midrib and not on the other side, so that the effect of an injected substance can be watched by comparing a leaflet on each side of the midrib.

Two leaves (Nos. 2 and 3) may be permeated in this manner by injecting a basal leaflet of the second leaf below them; this method therefore seems preferable to the leaf-stalk one. The interveinal method is to be preferred to either when the most rapid response is sought, because tissues separated only by a vein are more easily compared than separate leaflets on opposite sides of a comparatively thick midrib.

(b) *Experiments with nutrients.* (i) *Nitrogen.* A fairly mature plant, which had reached an advanced stage of nitrogen deficiency, shown by cessation of growth and yellow-green foliage, was injected by cutting off half the leaf-stalk of a basal leaf and dipping the cut end in 0.25 per cent. solution of ammonium nitrate for a few hours. After nine days the following results were obtained:

*Leaf 1:* The entire leaf was still a uniform greenish-yellow. *Leaf 2:* The leaflets on the side of the leaf nearer the injected leaf-stalk was slightly darker green, while the leaflets on the other side were unchanged. The nearer side of the terminal leaflet was darker green, while the far side was unchanged. *Leaf 3:* The entire leaf was darker green, but the leaflets on the nearer side were darker green than the leaflets on the farther side. *Leaf 4:* The entire leaf was still a uniform greenish-yellow. *Leaf 5:* The entire leaf was somewhat darker green. These results are in agreement with the distribution obtained by injection of the dye.

Another, less mature, plant which had not reached such an advanced stage of nitrogen deficiency was injected in a like manner with 0.5 per cent. ammonium nitrate. Following this injection, no localized effect was observed, but in seventeen days the entire foliage had attained a darker green and three new healthy green leaves had unfolded.

The difference in behaviour of these two plants following injection may be due to their relative differentiation of tissues. It has been shown that with interveinal leaf injections of the tomato relatively immature veins did not prove a barrier to distribution, while more mature veins restricted distribution. On the other hand, Roach (1938*a*) has observed a similar phenomenon in the Japanese plum, in which the majority of shoots give a definite 'injection pattern', but a few give a general distribution apparently as a result of an unusual arrangement of the strands. Further work is necessary on the potato.

(ii) *Phosphorus*. At the time of injection the plant was stunted, the leaves arising at a narrow angle with the stem and being somewhat small and dark green. No effect on foliage colour was observed following injection of 0.25 per cent. disodium hydrogen phosphate, but the plant renewed growth, the internodes became longer, and the plant became more vigorous and normal. Eighteen days after reinjection by the same leaf-stalk, the leaflets on the nearer side of leaf 2 were larger than the leaflets on the opposite side. The leaflets on leaf 4 were much smaller and less healthy in colour than those of leaf 5.

(iii) *Potassium*. A plant was injected with 0.25 per cent. solution of potassium sulphate when the foliage was crinkled and dark bluish-green. Twelve days after injection an absence of crinkling and a slight improvement in colour was observed on the second, third, fifth, and one side of the sixth leaf above that injected. No improvement was noted on leaf 4. Seventeen days after injection the tips and margins of leaf 4 had become chlorotic and dark brown spots had developed between the veins. Twenty-four days after injection the deficiency symptoms were apparent on all but the unfolding leaves at the growing tip.

Two plants in the same pot exhibited rolling and chlorosis of the tips and margins of the lower leaves, the upper leaves being light green. One of these plants was injected through the leaf-stalk of the first basal unaffected leaf with equal volumes of 0.25 per cent. solution of potassium chloride and 0.25 per cent. solution of potassium sulphate. Seventeen days after injection the injected plant had increased 3 cm. in height, and the deficiency symptoms had progressed no farther. The uninjected plant had made no growth and the margins of practically all the leaves were chlorotic, with severe brown patches between the veins.

Another plant affected with yellowing of the margins of the lower leaves and brown patches between the veins was injected with equal volumes of 0.25 per cent. solution of potassium chloride and 0.25 per cent. solution of potassium sulphate. The effects observed seven days after injection are seen in Plate II, Fig. 8, in which actual photographs of leaves have been placed on a phyllotaxis diagram; this may be compared with Text-fig. 4. The leaflets on the nearer side of leaf 2 above the one injected were healthy green, while the margins and tips of the leaflets on the other side were chlorotic. Leaf 3 was a healthy green, while the margins and tips of all the leaflets of leaf 4

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were distinctly chlorotic with brown patches between the veins. Leaf 5 was healthy green, while leaf 6 was not fully expanded. This result is therefore in full agreement with that obtained by injection of dye.

### THE BROAD BEAN

#### 1. *Cultural notes.*

Seeds were germinated and the plants grown in sand in 4-in. paraffined clay pots. The same nutrient solutions were employed as for tomato. Each pot was given 200 c.c. of nutrient solution once a week.

#### 2. *Deficiency symptoms.*

(i) *Nitrogen.* The leaves of nitrogen-deficient plants became a progressively lighter yellowish green colour.

(ii) *Phosphorus.* Phosphorus-deficient plants were stunted in growth, the leaves small and narrow and dull greyish-green.

(iii) *Potassium.* Deficiency symptoms were a shrivelling, followed by dark brown 'burning' and dropping of the basal leaves, the upper leaves becoming somewhat light green.

#### 3. *Interveinal injections.*

(a) *Experiments with dye.* Experiments with dyes proved that the secondary veins of broad bean leaves serve as efficient barriers to the lateral distribution of dye by interveinal injection. If absorption is allowed to proceed until the capillary tube is emptied there is a small amount of escape just along the leaf margin, but permeated and untreated tissues are separated sharply along most of the length of the secondary veins.

(b) *Experiments with nutrients.* (i) *Nitrogen.* Three yellowish-green leaflets were injected each with 0.25 per cent. ammonium nitrate, 0.25 per cent. sodium nitrate, and 0.5 per cent. urea respectively. Six to eight days later the injected interveinal areas had become distinctly darker green than the rest of the leaf, as shown in Plate II, Fig. 9.

(ii) *Phosphorus.* When the leaves of phosphorus-deficient plants had attained a dull greyish-green, interveinal injections were made with a 0.25 per cent. solution of disodium hydrogen phosphate. Seven days later there was a very slight change in colour of the injected interveinal areas, but not sufficiently intense to be of definite value for diagnostic purposes.

(iii) *Potassium.* No results were observed from interveinal injections with 0.25 per cent. potassium sulphate.

#### 4. *Leaflet-tip injection.*

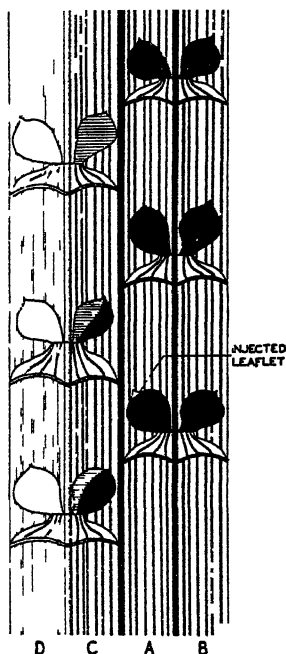
(a) *Experiments with dyes.* The leaves of the broad bean are arranged bilaterally on the four-sided stem with the base or basal sheath of the leaf occupying one-half of the stem circumference. Several of the basal leaves

develop two leaflets only. When the tip of one member of such a pair of leaflets was cut off and the cut end immersed in dye the other leaflet of the same leaf was first permeated. The dye then entered the main stem and permeated the nearer of the two leaflets of the first leaf below. The first and third leaves above the one injected and on the opposite side of the stem had only one each of the pair of leaflets permeated, while the second and fourth leaf above, on the same side of the stem, were entirely permeated.

The cause of such a distribution was seen on peeling off the external tissues from the stem to expose the vascular strands. In Text-fig. 6 the stem is represented as being opened and flattened out with the leaves left in position, the four sections A, B, C, D corresponding to the four sides of the stem. The leaf bases extend over two adjacent sections, the dividing-line of which is a ridge of greatly thickened strands. The heavily permeated strands are represented by solid black bands, lightly injected strands are hatched, and unaffected strands are left clear. The leaves or portions of leaves lightly and heavily permeated by dye are hatched or represented black respectively. Three strands enter the stem from each leaflet. The strands from the injected leaflet are very heavily permeated and consequently the strands in section A of the stem are heavily permeated. The strands from the other leaflet of the pair are less heavily permeated and the right-hand strands of section B of the stem are only lightly permeated. The dye is able to pass laterally through the band of strands dividing sections A and C, and permeate the strands of section C, thus permeating the leaflets supplied by these strands. The band of strands between sections B and D is not permeated, and the extreme left-hand strands between C and D remain unaffected, consequently the strands in section D of the stem also remain unaffected, and the leaflets supplied by these strands are not permeated.

This method, therefore, seems to hold out the possibility of a clear comparison between permeated and untreated tissue in paired leaflets (in stem sections C and D) and should be useful for diagnosis.

Leaves farther up the stem have two pairs of lateral leaflets and a terminal leaflet. When the tip of a lateral leaflet was cut off and the cut end immersed in dye all the leaflets of the injected leaf were permeated in 3-4 hours. After twenty-four hours the dye had passed into the stem and permeated



TEXT-FIG. 6. Distribution of dye in the broad bean, resulting from leaflet-tip injection of one member of a pair of leaflets of a basal leaf.

the leaflets on one side of the first leaf above all the leaflets of the second leaf below.

### 5. *Leaf-stalk injection.*

(a) *Experiments with dyes.* When injections were made through a cut leaf-stalk the foliage of the entire plant was permeated. It is evident from an examination of Text-fig. 6 that all the leaf traces connected with the strands of two sections of the stem should be heavily permeated and lateral movement should occur between sections A and C and between B and D. Leaf-stalk injection suggests itself as a means of treating a whole plant.

(b) *Experiments with nutrients.* (i) *Nitrogen.* A plant with yellowish-green foliage was injected with 0.25 per cent. sodium nitrate solution. In nine days a slight intensification of colour was observed in all the leaves.

(ii) *Phosphorus.* Plants showing definite signs of phosphorus deficiency were injected by cutting the leaf-stalk half-way and dipping the cut end in 0.25 per cent. solution of disodium hydrogen phosphate. In eight days there was a slight improvement in foliage colour. Nine days after injection the internodes had lengthened and the foliage was a more normal green colour.

(iii) *Potassium.* Plants showing potassium deficiency symptoms were injected with equal volumes of 0.25 per cent. potassium chloride and 0.25 per cent. potassium sulphate solutions. Nine to fourteen days later the upper leaves had attained a darker green and the upward progression of symptoms was checked.

## SUMMARY

1. A study was made of the distribution resulting from the injection of an aqueous solution of acid fuchsin into (a) an interveinal area of a leaf blade, (b) the leaf-tip, and (c) the leaf-stalk of the tomato, the potato, and the broad bean.

2. The plants were grown in sand culture deficient in one or more of the elements: nitrogen, phosphorus, potassium, calcium, magnesium, and boron, and were injected with solutions containing the deficient element.

3. The plants responded to all the elements injected.

4. The regions of the plants which responded to the nutrient injections were similar to those coloured by the dyes (par. 1, above).

5. The response was local or general according to the injection method used.

6. The distribution of the injected liquids could be explained by the arrangement of the vascular system of the plants.

7. The methods described, (a) enable diagnosis of deficiencies to be made in 7-21 days, and (b) provide means of rapidly, and without injury, making good any deficiency to which the plant has been subjected.

# ACKNOWLEDGEMENTS

One of us (H. H.) is indebted to Dr. R. G. Hatton, Director of the East Malling Research Station, for the facilities placed at his disposal. Acknowledgements are due to Miss K. Cornford for assistance in photography.

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# EXPLANATION OF PLATES II, III, IV

Illustrating Dr. Hill and Dr. Roach's paper on 'Injection for the Diagnosis of Mineral Deficiencies in the Tomato, the Potato, and the Broad Bean'.

## PLATE II

Figs. 1, 2, 3. Distribution of acid fuchsin in tomato leaflets of increasing size by interveinal injection. Arrows o indicate injection incisions.

Fig. 4. Retardation of boron deficiency symptoms in tomato leaf obtained by interveinal injection with 0.001 per cent. boric acid.

Figs. 5, 6, 7. Distribution of acid fuchsin in potato leaflets of increasing size by interveinal injection. Arrows o indicate injection incisions.

Fig. 8. The effect of leaf-stalk injection of a potassium-starved plant with equal volumes of 0.25 per cent. solution of potassium chloride and 0.25 per cent. solution of potassium sulphate. The non-permeation of leaf 4 and half of leaf 2 is reflected by the occurrence in them of deficiency symptoms.

Fig. 9. Nitrogen-starved broad bean plant: intensification of colour in a band of tissue noted six days after interveinal injection with 0.25 per cent. solution of urea.

## PLATE III

Fig. 10. Tomato plant injected with 0.25 per cent. urea. Fig. 11 not injected.

Fig. 12. Tomato plant twenty-seven days after injection with 0.25 per cent. ammonium nitrate. Fig. 13 not injected.

Fig. 14. Tomato plant injected with 0.25 per cent. disodium hydrogen phosphate. Fig. 15 not injected.

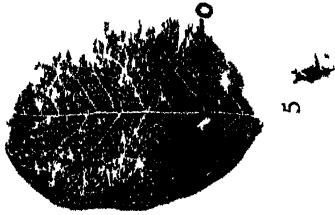
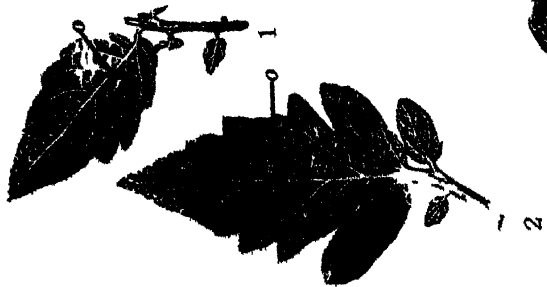
## PLATE IV

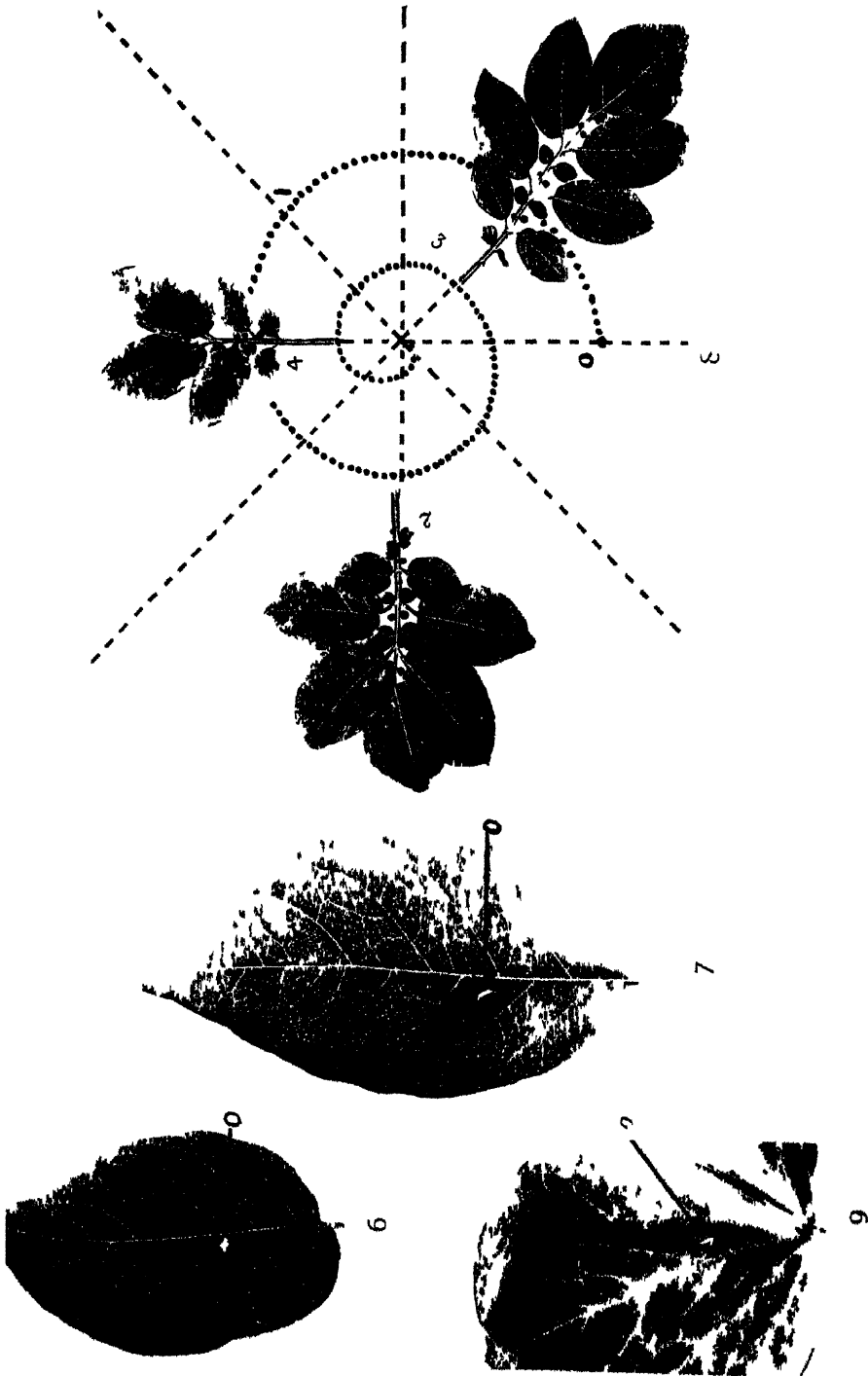
Fig. 16. Tomato plant injected periodically with 0.25 per cent. potassium sulphate. Fig. 17 not injected.

Fig. 18. Tomato plant injected with 0.25 per cent. potassium sulphate after appearance of deficiency symptoms on the lower leaves. Fig. 19 not injected.

Fig. 20. Tomato plant injected periodically with 0.001 per cent. boric acid. Fig. 21 not injected.







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# On the Xylem Elements of Certain Ferns

BY

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With Plate V

THE problem of the real nature of the xylem elements in Pteridophytes generally, centres in the presence or absence of the pit-closing membrane, which, when present, halves the pit cavity. On this subject the earlier anatomists held conflicting views. Some, as Valentin (1836), believed that the membrane was always present. Others, for example Dippel (1800) and Sachs (1873), denied its existence, at least in mature xylem. Russow in 1872 figured the xylem of *Botrychium rutaefolium* showing no closing membrane in the pits, neither on the lateral nor the end walls, and also recorded the occurrence of end walls with truly perforated pits in *Pteridium aquilinum* and in the root of *Athyrium Filix-foemina*. In 1877 de Bary demonstrated the pit-membrane in some vascular cryptogams and in particular in *Pteridium aquilinum*, in the pits of the lateral walls.

At the time of the publication of Gwynne-Vaughan's work in 1908 the generally accepted belief was in the persistence of the pit-closing membrane and therefore in the tracheidal nature of the xylem in vascular cryptogams, with the occurrence of vessels in the exceptional cases of *Pteridium aquilinum* and the root of *Athyrium Filix-foemina*. Gwynne-Vaughan found that in the material he examined the xylem elements were vessels with true perforations on both longitudinal and transverse walls, the pit-closing membrane having disappeared in the mature wood. Moreover, he found that in the Osmundaceae, in *Dryopteris Filix-mas*, and probably others, a special type of vessel occurred with complete disappearance of the primary tracheal wall at certain points, so that the cavities of the pits are vertically continuous in the middle of the wall. On the other hand, Gwynne-Vaughan found that in *Pteridium aquilinum* the pit-membrane disappears between the cavities of the pits, but the transverse bars of secondary thickening are intact. Vessels of the latter type he found also in various members of the Polypodiaceae, Hymenophyllaceae, Gleicheniaceae, Schizaeaceae, Marattiaceae, Ophioglossaceae, Lycopodiaceae, and in the fossils Psaronius, Botryopteris, and Zygopteris, but true tracheides in Calamites.

In 1910 Hialft demonstrated the pit-membrane in both end and side walls of the xylem of vascular cryptogams he examined, with the exception of

*Pteridium aquilinum*, in which the membrane was absent from the pits of the end walls, but not from those of the side walls as was stated by Gwynne-Vaughan (1908). In *Osmunda regalis* Halft found that the xylem was tracheidal and not of the special vessel type described by Gwynne-Vaughan (1908) for this fern.

A year later Bancroft (1911) found that the pits of both side and end walls of the xylem of Pteridophyta she examined were closed by membranes formed by the persistent mid-lamella, and that such elements were therefore tracheides. Wright in 1920 showed that the pit-membrane was present between the pits in the xylem of members of the Ophioglossaceae, and demonstrated the occurrence of a torus in Botrychium and in Helminthostachys.

Halft's observations upon *Pteridium aquilinum* were supported in 1929 by Meyer, who described true perforations in the oblique end walls of the xylem of this form.

In 1933 the author observed the tracheidal nature of the xylem in petiolar material of *Metaclepysdropsis duplex* and *Diplolabis Römeri*, and the absence of the pit-membrane in petiolar xylem of *Stauropteris burntislandica*. Further, in 1934 the occurrence of true vessels in the xylem of several homophyllous species of Selaginella.

From the work of Gwynne-Vaughan (1908) and Halft (1910) in particular, we have thus two opposing conceptions of the nature of the xylem elements in the Pteridophyta, and the critical observations appear to have been centred in the Osmundaceae, *Dryopteris Filix-mas* and *Pteridium aquilinum*.

In order to clear up this duality of conception, attention is confined in the present work to the detailed structure of the xylem elements in a few particular cases of living ferns.

In the preliminary treatment fresh material was always cut into short lengths, and in all possible cases the hard cortical tissues were dissected away before fixation. The fixatives used were 95 per cent. alcohol, medium chromo-acetic, and Farmer's fluid (acetic alcohol). After washing, all material was submitted to a softening process before embedding. In the earlier stages of the work the cellulose-acetate method described by Williamson (1921) was used for this purpose. The material was placed in pure acetone for two hours and then transferred to a 12 per cent. solution of cellulose-acetate in acetone and left in this solution for fourteen days. At the end of this time it was transferred to acetone for three hours, and from this point the procedure given by Larbaud (1921) was followed. That is, the material was placed in 95 per cent. butyl alcohol, and then through two baths of pure butyl alcohol, to the second of which paraffin was added, preparatory to embedding. In most of the work with the cellulose-acetate method of softening, however, after the three-hour bath in pure acetone, the material was transferred to a mixture of acetone and alcohol in equal parts, from this into absolute alcohol, and then by a graded alcohol chloroform series into pure chloroform, to which the wax was added.

In the later work Calberla's fluid was employed as a softening medium. After fourteen days in this fluid the material was washed in 95 per cent. alcohol,

then in absolute alcohol, and passed in the usual way into chloroform. Wax of 52° C. melting-point was used in all cases for embedding. After pouring into the embedding dish the wax was hardened rapidly in iced water.

Transverse and longitudinal sections were cut at  $4\mu$  to  $10\mu$  in thickness. Sections of the material which had been softened in the cellulose-acetate solution were washed in pure acetone for two minutes in order to remove any trace of the cellulose acetate. They were then washed in absolute alcohol before passing on to the staining process.

A very good stain for the primary substance of the walls and the pit-closing membrane was found to be ruthenium red in aqueous solution rendered slightly alkaline with ammonia. Methylene blue was used as a counter stain. Good results were also obtained with Heidenhain's iron haematoxylin. Delafield's haematoxylin was also used, with safranin as a counter stain. The best results were, however, given by using gentian violet.

A chemical method used by Halft (1910) was tried with transverse sections of certain forms. The sections were treated with Schultze's macerating fluid to remove the lignin of the secondarily thickened bars, leaving a residue of cellulose, then concentrated sulphuric acid was added to destroy the cellulose, with the result that the pit-closing membranes and primary walls remain as a fine network on the slide.

An examination of both stem and root xylem of *Dryopteris Filix-mas* (L.) Schott was made. In the case of the stem xylem no evidence was obtained of the disappearance of the pit-membrane from either the vertical or the end walls of both young and old xylem, the elements being entirely tracheidal. Pl. V, Fig. 1, illustrates the appearance of several pits in transverse section; the membrane is distinctly visible in each of the pits, in some cases following a wavy course in the slit between the paired secondary bars and continuous at each corner with the triangular pieces of primary wall at the angles. The whole thickness of the primary wall is shown at two places in this figure, where in each case a parenchymatous cell abuts upon a tracheide and the secondarily thickened layer has only been developed on the tracheide side of the primary wall at these points.

In the longitudinal sections of the side walls the membrane can always be demonstrated in the pits, as shown in Pl. V, Fig. 2, and Pl. V, Fig. 3. The latter figure also shows the membrane in the pits on an oblique end wall. In this case there has been some displacement of the secondary bars in cutting the section, but the delicate membrane may be traced as a fine connecting line along one series of the secondary bars.

The root xylem of this fern also appears to be tracheidal. On the vertical walls the pit-closing membrane can always be seen (Pl. V, Fig. 4). The oblique end walls also show closed pits (Pl. V, Fig. 5).

In the case of *Pteridium aquilinum* (L.) Kuhn both very young and very old xylem of rhizome and petiole was investigated. Vertical sections passing through the side walls of the elements show the thickened secondary bars cut



through with the primary cementing material between each pair of secondary bars and the pit-membrane extending across each pit cavity, Pl. V, Fig. 6. The oblique end walls, however, show clear perforations, the pit-closing membrane having disappeared (Pl. V, Fig. 7). It is clear that the membrane disappears from these oblique end walls at an early stage in development, for the section figured here is of petiolar material which was collected very early in the season when the fronds were just appearing above the ground.

Pl. V, Fig. 7, illustrates very clearly the large size of the open pits on the oblique end walls compared with the small pit apertures of the closed pits on the vertical wall (Pl. V, Fig. 6), the latter being about  $0.5\mu$  only, whilst the former range from  $2.0\mu$  to  $2.5\mu$  for the most part, but narrow down to  $1.0\mu$  at each end of the oblique wall, where it becomes continuous with the vertical walls on each side. Examination of vertical sections of the xylem of old rhizome material revealed that, although true perforations were present on the oblique end walls, yet between the pits on the side walls the closing membrane was always present, and, as in the case of the petiolar xylem, the persistent piece of primary wall was always present between each pair of secondary bars.

The pit apertures were found to be larger in the xylem walls of the rhizome than those of the petiole, those of the side walls being about  $1.0\mu$  in longitudinal section of the wall, and those of the oblique end walls from  $3.0\mu$  to  $4.0\mu$  in corresponding section. Another point of difference between the petiole xylem and the rhizome xylem appears to be in the inclination of the end walls. In the former the end walls of the vessels are more sharply inclined to the side walls than are those of the vessels of the latter. Pl. V, Fig. 8, shows a piece of rhizome xylem in transverse section and the pit-membrane may be seen stretching along the pit cavity between the paired secondary bars, which, on the vertical walls, are comparatively thick and stout, in this respect contrasting sharply with the thinner and more delicate bars of the oblique end walls, one of which is shown in transverse section in Pl. V, Fig. 9. Here no membrane can be seen, there being a clear slit between the thin secondary bars.

The xylem elements of both petiole and rhizome of *Pteridium aquilinum* are therefore in open communication with each other vertically, the pits on the oblique end walls being true perforations and the elements true vessels, but the closing membrane is always present in the pits of the vertical walls.

An investigation of the root xylem of *Athyrium Filix-foemina* (L.) Roth. provided no evidence of perforated pits on the end walls of the elements. Longitudinal sections always showed the closing membrane in the pits on the oblique end walls and also on the side walls. The membrane was also plainly seen in both types of wall in transverse sections of the root xylem. The stem xylem of this fern, however, showed some true vessels. A delicate membrane is always present in the pits on the side walls, but in some cases the end walls showed pits with true perforations (Pl. V, Fig. 10).

In *Todea barbara* Moore the pit-membrane was observed in sections from all parts of the stem, with the exception of one short length of a vertical wall

illustrated in Pl. V, Fig. 11. Here, although an extremely delicate membrane may be seen in one or two of the pits, yet others appear to be perforated. At only a short distance from this point the same wall shows a definite membrane in each pit. An extensive examination of much material failed to furnish any additional instances of clear pits on any of the walls, even in old xylem. The pit-closing membrane was always present (Pl. V, Fig. 12), or, if not the membrane alone, then a thick vertical wall representing the whole of the primary wall, which in such cases has evidently persisted in the pit areas as well as between the secondarily thickened bars (Pl. V, Fig. 13). This feature of wall structure is seen in mature and old xylem as well as in young xylem. It would then appear to be a persistent feature of some of the vertical xylem walls of this fern. Moreover, staining reactions indicate that this persistent wall is lignified, for it stains definitely with methylene blue, safranin, and phloroglucin and hydrochloric acid. In order to be certain that this condition was not peculiar to one plant, the stem xylem of other plants of *Todea barbara* was examined and the same feature was found in each case on some of the walls.

Walls of the type just described may be distinguished in transverse sections of the xylem as solid walls. Pl. V, Fig. 14, shows such walls in the lower middle and right-hand portions of the figure. Serial transverse sections show that such walls remain solid through successive sections. In Pl. V, Fig. 14, the pit-membrane may be seen as a fine line in several pits to the left of the figure.

The stem xylem of all ages of *Todea barbara* was thoroughly examined, but, apart from the isolated instance of the few apparently clear pits on the side wall illustrated in Pl. V, Fig. 11, no further examples of this kind were found, neither on vertical nor on oblique end walls, nor in transverse sections could any pits be found without the membrane.

The cumulative evidence from transverse and longitudinal sections of stem xylem of *Todea barbara* points to its tracheidal nature. In addition, in some walls the whole of the primary wall persists and is lignified, both between the secondary bars and in the pit areas, and forms a continuous middle wall (Pl. V, Fig. 13).

In transverse sections of young and old stem xylem of *Osmunda cinnamomea* L. the pit-membrane shows clearly in each pit. The vertical sections, however, provide a difficulty. The usual appearance of the walls is shown in Pl. V, Fig. 15, where, although there has been slight displacement of the tissues in cutting them, both the membrane in the pits and the primary cementing portions of wall between the paired secondary bars are present. Some walls are found, however, which show the condition illustrated in Pl. V, Fig. 16, where neither the primary tracheal wall between the paired secondary bars nor the pit-membrane is to be seen, so that here are apparently perforated pits which are vertically continuous in the middle of the wall.

Thus, in *O. cinnamomea* transverse sections of the stem xylem show the pit-membrane always present whilst the vertical sections yield conflicting evidence: in general the walls are intact with closing membranes in the pits, as

shown in Pl. V, Fig. 15, but more rarely apparently hollow walls of the type illustrated in Pl. V, Fig. 16, are found, the latter condition being exactly that stated by Gwynne-Vaughan (1908) to occur in the xylem of the Osmundaceae, and which has also been described by the author (1932).

Then chemical tests on the lines described by Halft (1910) were made. Transverse sections of the xylem of *O. cinnamomea* were treated with Schultze's macerating fluid (concentrated nitric acid and potassium chlorate) in order to remove the lignin of the secondarily thickened bars, leaving a residue of cellulose. Concentrated sulphuric acid was then added to destroy the cellulose, the pit-closing membranes and primary walls remained for a short time as a fine network on the slide. Three stages in this process are shown in Pl. V, Figs. 17, 18, and 19. In the first of the figures the appearance of the xylem walls is represented when the sulphuric acid has just commenced action, the secondarily thickened bars are beginning to swell before disintegration and the pit-membranes are clearly visible. The second figure shows a more advanced stage in the disintegration of the secondary bars; the primary portions of the walls and the pit-membranes are very distinct. In the third figure is shown complete disintegration of the secondary bars, leaving for a little time the primary portions of the walls and the pit-membranes as a network.

The xylem of *Osmunda regalis* L. was examined and showed all the features which have just been described for *O. cinnamomea*, including a similar reaction to the chemical test. The collective evidence of transverse and vertical sections of the xylem in these two members of the Osmundaceae points to the presence of the pit-closing membrane. The difficulty presented by lengths of wall of the type shown in Pl. V, Fig. 16, will be discussed in the following section.

### CONCLUSION

Gwynne-Vaughan's observations (1908) on the special nature of the xylem elements in *Dryopteris Filix-mas* are not borne out by the present investigation. The primary portion of the wall is present between the paired secondary bars and the pit-membrane is present in the pits on both vertical and oblique walls of root and stem xylem, that is, the xylem of this fern is entirely tracheidal.

Confirmation of the views of Russow (1872), de Bary (1877), Halft (1910), and Meyer (1929), is provided by the result of the work on the petiole and rhizome of *Pteridium aquilinum*. Transverse and vertical sections show the xylem elements to be true vessels, with open pits on the end walls only, not on the side walls as was held by Gwynne-Vaughan (1908).

Russow's statement regarding the occurrence of true perforations on the end walls of the root xylem in *Athyrium Filix-foemina* was not confirmed, but sections of the stem xylem showed that these were occasionally present there (Pl. V, Fig. 10).

Young and old stem xylem of *Todea Barbara* is tracheidal. Apart from the apparent perforations in a few pits on one short length of vertical wall

illustrated in Pl. V, Fig. 11, the pit-closing membrane was seen in all vertical and oblique walls, with the special modification that on some of the vertical walls the whole of the primary wall has persisted and become lignified (Pl. V, Fig. 13).

Examination of the stem xylem of *Osmunda cinnamomea* and *O. regalis* furnished identical results. Transverse sections clearly show the membrane in all the pits on all the walls. In longitudinal sections the great majority of vertical and oblique walls show the paired secondary bars to be intact and the pit-closing membrane in the pits (Pl. V, Fig. 15), but some lengths of wall show neither pit-membrane nor the primary portion of the wall between each pair of secondary bars. Such lengths of wall, in addition to being apparently perforated laterally by the absence of closing membranes, are also apparently hollow in the middle of the wall, due to the absence of the cementing pieces of primary wall. If in transverse sections pits could be found with clear slits between the secondary bars, then the occurrence of walls with open pits and vertical continuity in the middle of the wall could be accepted as a real condition of some, at any rate, of the walls, but no such appearance in transverse sections is ever found. The stained transverse sections show the membrane in each pit. Furthermore, the chemical test with Schultze's maceration fluid and strong sulphuric acid yields a pit-membrane for each pit on every wall: this would not be the case were the walls hollow, without membranes and primary pieces between each pair of secondary bars, as would appear from such sections as figured in Pl. III, Fig. 16.

The making of transverse sections, sufficiently thin, of this type of xylem provides no difficulty, but thin longitudinal sections are much harder to get without occasional displacement and springing apart of the bars, even after softening treatment; and it is suggested that, as confirmatory evidence in transverse sections is never found, the exceptional appearance of walls perforated laterally and vertically when seen in vertical sections (Pl. V, Fig. 16) is probably due to breakage and loss in these particular walls during the cutting of the sections. Thus, the sum of the evidence for the three members of the Osmundaceae examined in this work indicates that the xylem is tracheidal in nature.

#### SUMMARY

The xylem elements of certain living ferns have been examined.

In *Dryopteris Filix-mas* (L.) Schott the stem and root xylem consists wholly of tracheids.

True perforations are found in the pits on the oblique end walls of the xylem elements of the petiole and the rhizome of *Pteridium aquilinum* (L.) Kuhn, these elements therefore being true vessels.

The root xylem of *Athyrium Filix-foemina* (L.) Roth. is tracheidal, but some true vessels are found in the stem.

In the Osmundaceae, *Todea barbara* Moore shows tracheidal xylem in the

stem. Moreover, in some of the vertical walls the whole of the primary wall persists and is lignified. The stem xylem of *Osmunda cinnamomea* L. and *Osmunda regalis* L. is tracheidal.

#### ACKNOWLEDGEMENTS

To Professor W. H. Lang I am indebted for valuable criticism, and to him also, and to Mr. Eric Ashby, and Mr. C. J. A. Berkeley for help in the photographic illustration of the paper. The work was carried out in the Department of Botany, Birkbeck College, and the Department of Biology, Chelsea Polytechnic, and has been aided by the use of a microscope obtained by means of a grant from the Dixon Fund of the University of London.

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#### EXPLANATION OF PLATE V

Illustrating Dr. H. Duerden's paper 'On the Xylem Elements of Certain Ferns'.

All figures are from untouched photographs

Figs. 1-5, *Dryopteris Filix-mas*.

Fig. 1. Transverse section of stem xylem showing the pit-membrane in several pits. ( $\times 1,270$ .)

Fig. 2. Longitudinal section of stem xylem showing the pit-membrane between the pits on both vertical and oblique end walls. ( $\times 1,270$ .)

Fig. 3. Longitudinal section of stem xylem showing the pit-membrane between the pits on both vertical and oblique end walls. ( $\times 1,270$ .)

Fig. 4. Longitudinal section of root xylem showing the pit-membrane in the pits on a vertical wall. ( $\times 1,270$ .)

Fig. 5. Longitudinal section of root xylem with the pit-membranes in the pits on an oblique end wall. ( $\times 1,400$ .)

Figs. 6-9. *Pteridium aquilinum*.

Fig. 6. Longitudinal section of xylem of young petiole showing pit-membranes in the pits on a vertical wall. ( $\times 1,400$ .)

Fig. 7. Longitudinal section of xylem of young petiole showing the wide pits with no closing membranes on an oblique end wall. In this figure the vertical wall, which is the same length of wall as that shown in Fig. 6, is just out of focus. ( $\times 1,400$ .)

Fig. 8. Transverse section of the rhizome xylem showing the thick secondary bars of a vertical wall with the pit-membrane in the narrow pit. ( $\times 1,090$ .)

Fig. 9. Transverse section of the rhizome xylem showing the thin secondary bars of an oblique end wall with no pit-membrane in the narrow pit cavity between them. ( $\times 1,090$ .)

Fig. 10. *Athyrium Filix-foemina*. Longitudinal section of the stem xylem showing open pits in an end wall. ( $\times 1,250$ .)

Figs. 11-14. *Todea barbara*.

Fig. 11. Longitudinal section of stem xylem, and vertical wall with some clear pits. ( $\times 1,400$ .)

Fig. 12. Longitudinal section of the stem xylem showing the pit-membrane in each of the pits. ( $\times 1,250$ .)

Fig. 13. Longitudinal section of stem xylem showing persistent and lignified primary wall between the paired secondary bars of a vertical wall. ( $\times 1,250$ .)

Fig. 14. Transverse section of the stem xylem showing the pit-membranes in the pits, and the persistent primary walls between the paired secondary bars, seen in the lower middle and right-hand parts of the figure. ( $\times 1,250$ .)

Figs. 15-19. *Osmunda cinnamomea*.

Fig. 15. Longitudinal section of a vertical wall of the stem xylem showing the pit-membrane in all the pits. ( $\times 1,250$ .)

Fig. 16. Longitudinal section of a length of vertical wall showing the absence of the cementing piece of primary wall between the members of each pair of secondary bars as well as the absence of the pit-membranes in the pits. ( $\times 1,250$ .)

Figs. 17-19. Transverse sections of stem xylem in strong sulphuric acid after treatment with Schultze's macerating fluid. (All  $\times 494$ .)

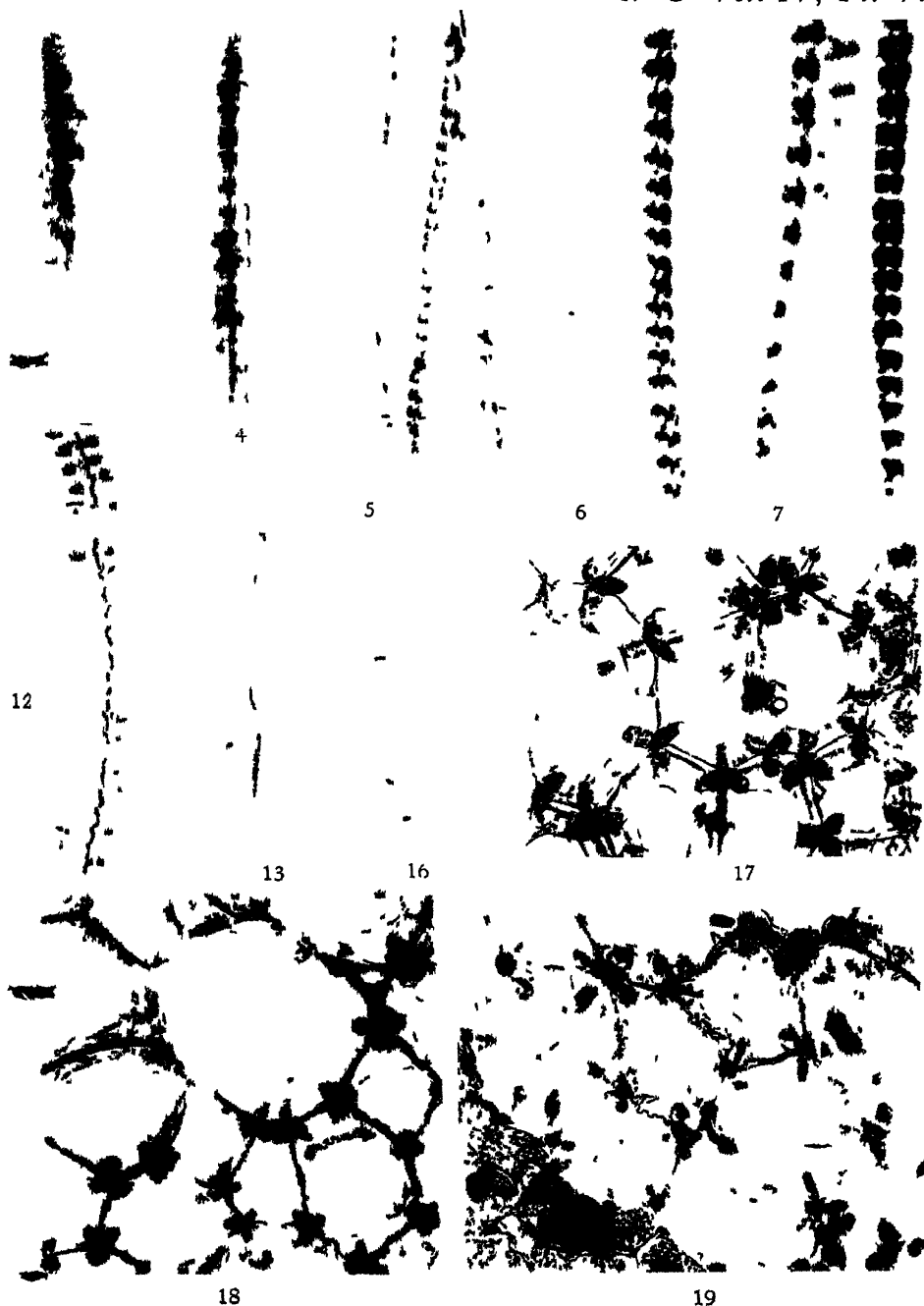
Fig. 17. Pit-membranes visible in the pits. Secondary bars beginning to swell.

Fig. 18. Secondary bars disintegrating, pit-membranes clearly visible.

Fig. 19. Primary portions of walls and pit-membranes remaining after disintegration of the secondary bars.



DURDEN - XYLEM ELEMENTS OF FERNS.







# The Interaction of Light Intensity and Nitrogen Supply in the Growth and Metabolism of Grasses and Clover (*Trifolium repens*)

## IV. The Relation of Light Intensity and Nitrogen Supply to the Protein Metabolism of the Leaves of Grasses

BY

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AND

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Chemical Industries Research Station, Warfield, Berks.)*

With 23 Figures in the Text

### INTRODUCTION

THE first of this series of papers (Blackman, 1938) was concerned with the effects of variations in the light intensity and nitrogen supply on the clover (*Trifolium repens*) content of a sward. It was shown that in full daylight the addition of calcium nitrate and more particularly ammonium sulphate depressed the clover, whereas at lower light intensities (0.6–0.4 of daylight) the effects of additional nitrogen were completely masked by the diminution of clover brought about by shading. Subsequently (Blackman and Templeman, 1938) a study was made of the influence of these light and nitrogen factors on the leaf production of grasses and *T. repens* when the plants were frequently defoliated. In full daylight it was found that both calcium nitrate and ammonium sulphate increased the leaf production of clover to a small extent and that of the grasses (*Agrostis tenuis* and *Festuca rubra*) by a large amount. At lower light levels, more particularly at an intensity of 0.4 daylight, the grasses and clover reacted very differently to additional nitrogen. In the case of clover, apart from the very marked effect of shading in lowering leaf production, neither ammonium sulphate nor calcium nitrate caused any appreciable change. When the grasses were shaded, however, the addition of nitrogen depressed the production of leaves and the depression was accentuated with each successive defoliation. Moreover, the somewhat surprising result was obtained that the diminution caused by calcium nitrate was greater than that brought about by ammonium sulphate.

In view of the claim of Prianishnikov (1910, 1922) that the accumulation of ammonia nitrogen produces deleterious effects and that such accumulation is associated with carbohydrate deficiency, it seemed clear that the results of



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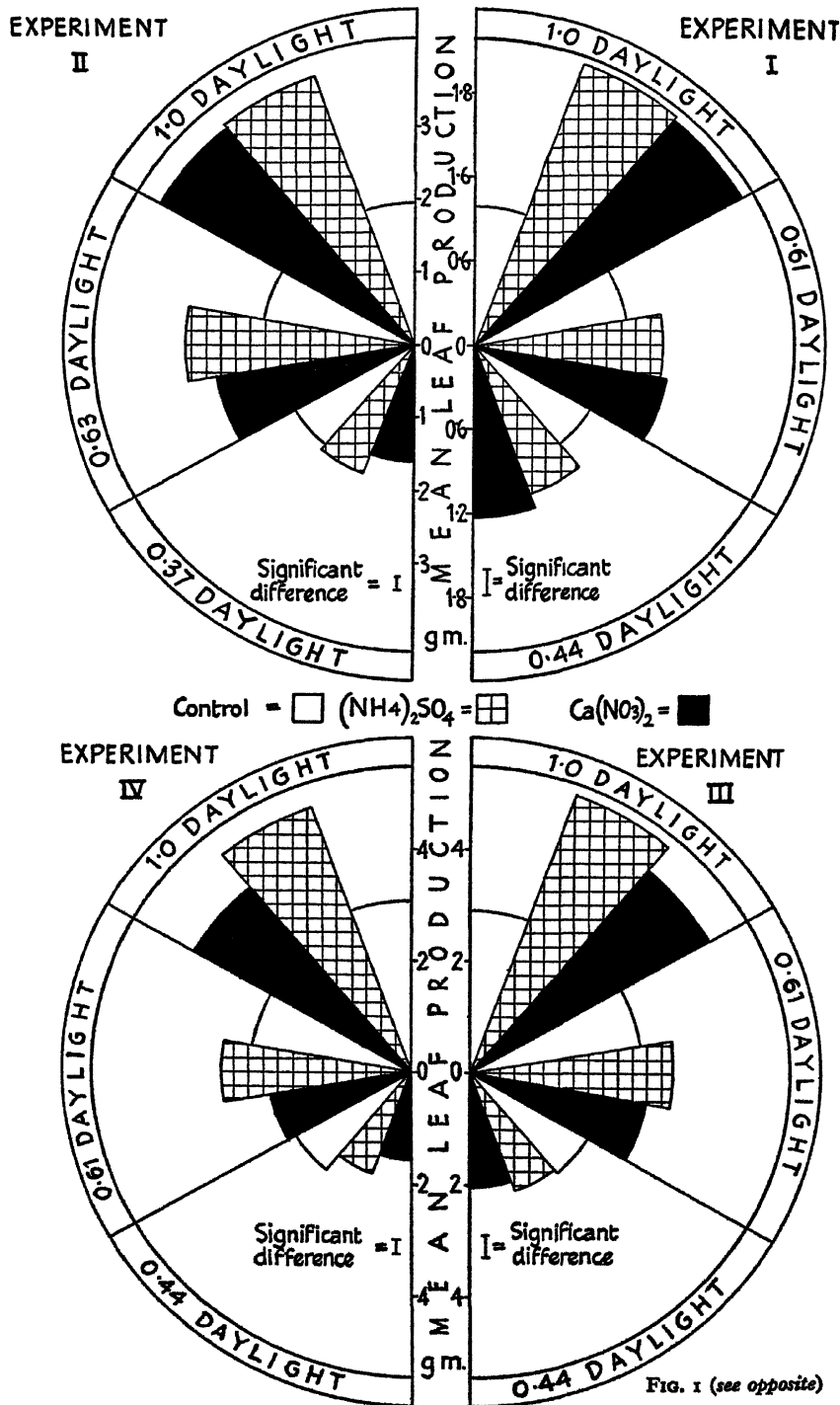


FIG. 1 (see opposite)

these two investigations could not be adequately interpreted without a study of the nitrogen and carbohydrate constituents of the plants. In the third paper (Blackman and Templeman, 1940) the methods employed for the estimation of the various carbohydrate and nitrogen fractions were described. The present paper is concerned with the analytical data obtained for the grasses; the results for *T. repens* will be given subsequently.

#### EXPERIMENTAL RESULTS

The analyses were carried out on the dried leaves obtained from four of the experiments of which the growth data have been described in the second paper of this series. In order, however, to interpret with greater clarity the present data the main results for these particular experiments will be recapitulated.

Three experiments were conducted in 1935, two with *A. tenuis* and one with *F. rubra*, while in 1936 a single experiment on *A. tenuis* was undertaken. In each case until the plants were established they were either not cut at all or cut but seldom. During the experimental period they were, however, defoliated some six to eight times at intervals of seven to fourteen days. The range of light intensity was obtained by shading the plants with screens of one or more layers of butter muslin while the additional nitrogen—either as ammonium sulphate or calcium nitrate—was added after each cut.

The main effects of light intensity and nitrogen supply on the mean leaf production are seen in Fig. 1. In all four experiments both calcium nitrate and ammonium sulphate at the highest light intensity (full daylight) increased leaf production. At the intermediate light level (0.61–0.63 of daylight) production is in part controlled by light, but nevertheless ammonium sulphate, except in experiment III, brought about an increase. However, in only one experiment (expt. I) does the calcium nitrate effect not differ from that of ammonium sulphate. In experiment III calcium nitrate has not raised leaf production, while in experiment IV it has depressed it. At the lowest light level (0.44–0.37 of daylight) these differences between the ammonium sulphate and calcium nitrate effects are again noticeable. Moreover, at this light intensity both calcium nitrate and ammonium sulphate have diminished leaf production, e.g. experiment IV. Thus the experiments fall into a series in which the interactions between light intensity and the effect of the two sources of nitrogen are negligible in experiment I and most pronounced in experiment IV. In the consideration of the analytical data this gradation from experiments I to IV will be stressed.

#### *The level of total nitrogen in relation to nitrogen supply and light intensity.*

In all experiments estimates of the total nitrogen content of the leaves were made on the samples collected at each cut. The results are shown graphically

FIG. 1. The effects of light intensity and of nitrogen supply on the mean leaf production of grasses when frequently defoliated. Experiments I, II, and IV, *Agrostis tenuis*; experiment III, *Festuca rubra*.

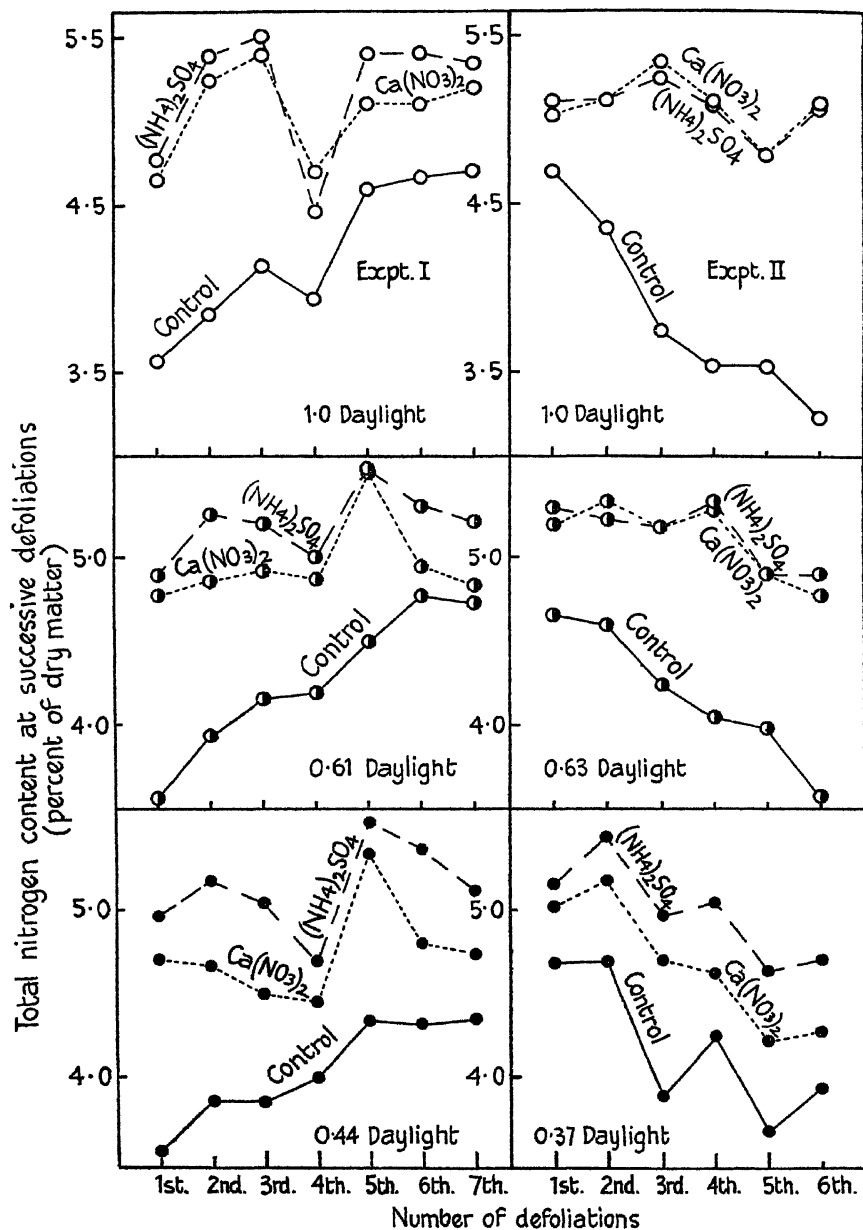


FIG. 2. The effects of light intensity and of nitrogen supply on the total nitrogen content of the leaves of *A. tenuis* at successive defoliations. (Experiment I, Aug. 14–Nov. 1, 1935; experiment II, May 8–July 9, 1936.)

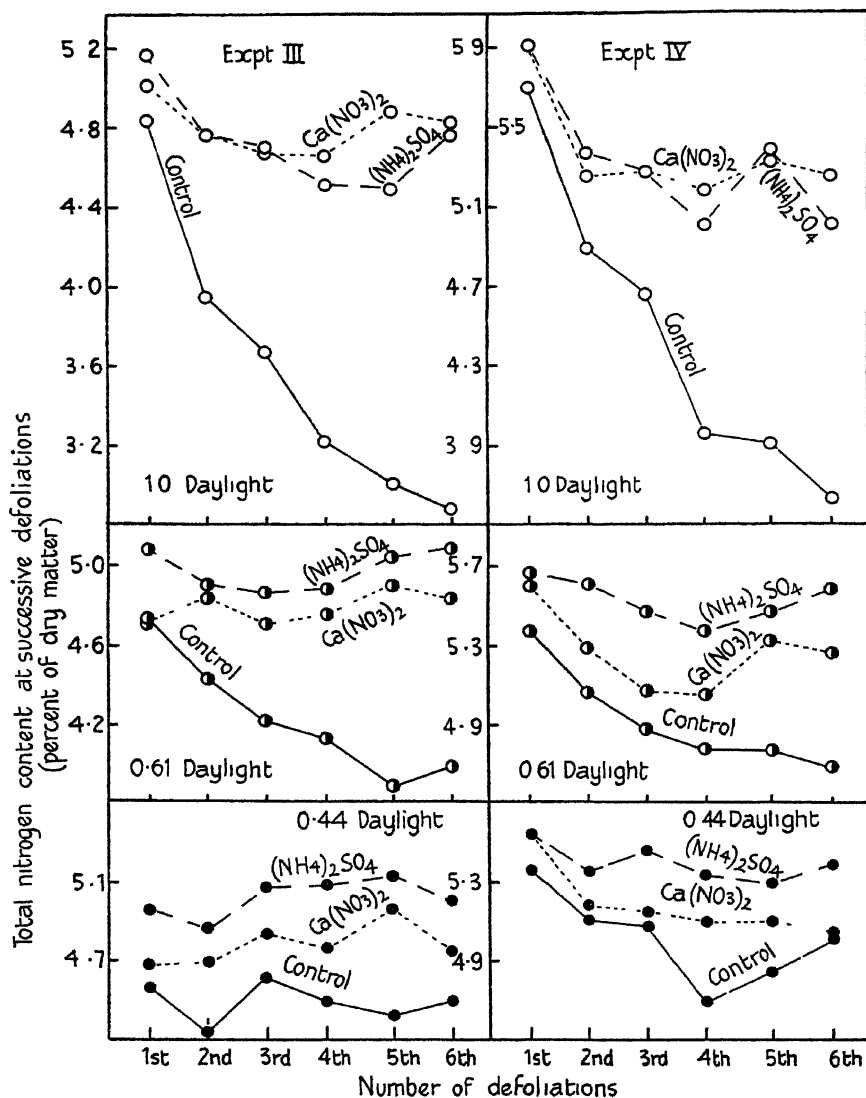


FIG. 3. The effects of light intensity and of nitrogen supply on the total nitrogen content of the leaves of *F. rubra* (experiment III) and *A. tenuis* (experiment IV) at successive defoliations. (June 18-Aug. 26, 1935.)

in Figs. 2 and 3 and the mean contents given in Table I. In the absence of additional nitrogen a decrease in the light intensity has led to an increased nitrogen content save in experiment I (Fig. 2) where, unlike in the other three



experiments, the total nitrogen content of the unmanured plants rises rather than falls with time. The marked accumulation of nitrogen in the leaves as a result of manuring (see Figs. 2 and 3) is dependent both on the number of defoliations and the light intensity. In full daylight the difference in the

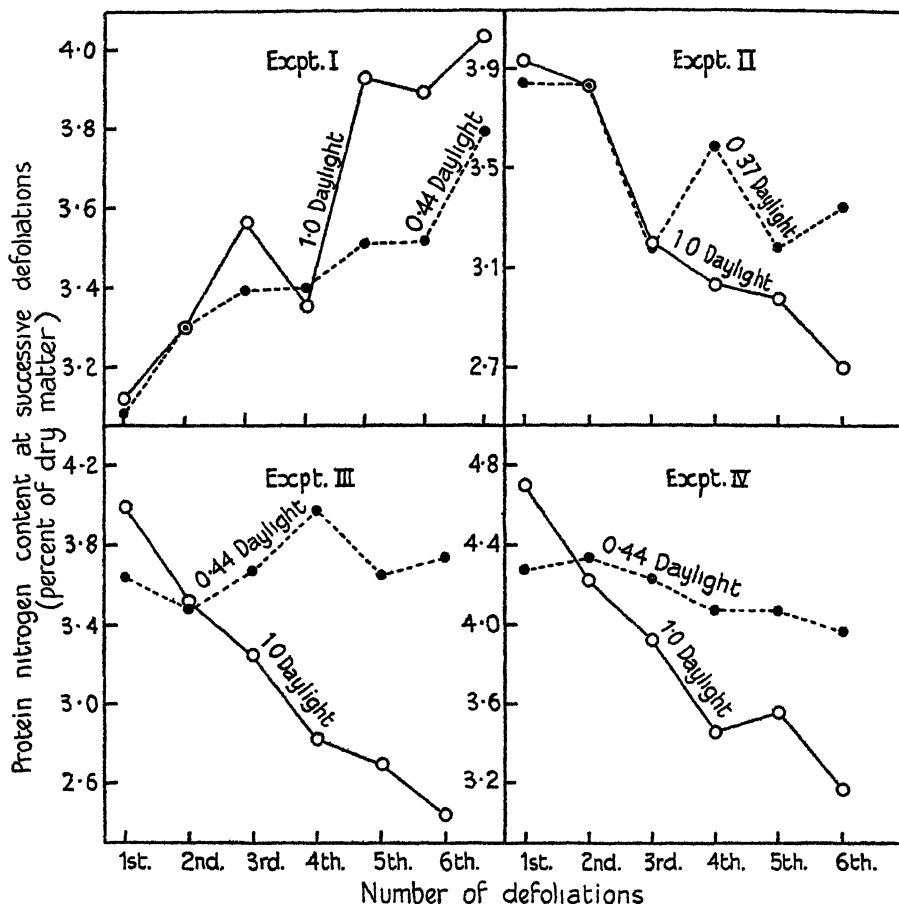


FIG. 4. The effects of light intensity on the protein nitrogen content of the leaves of control plants (low nitrogen supply) at successive defoliations. (Experiments I, II, and IV, *A. temuis*; experiment III, *F. rubra*.)

nitrogen content between the control and the manured plants becomes more pronounced with successive cuts in experiments II–IV (see also Figs. 6–8). But in experiment I (Figs. 2 and 5) the difference decreases with time. At the lowest light intensity this trend is less evident; in fact, except in experiment I, the gains in nitrogen content over the controls show if anything a negative correlation with frequency of cutting.

That the effects of high and low light intensity on the accumulation of

nitrogen in manured leaves are significantly dissimilar is seen in Table II. For the purpose of statistical analysis regression equations were first fitted to the data shown in Figs. 5-8, i.e. regressions of the difference from the control in nitrogen content against the number of defoliations. Subsequently the analysis of variance was carried out on the regression coefficients, the results for individual experiments being regarded as replicates.

TABLE I

*The Influence of Light Intensity and Nitrogen Supply on the Content and Amount of Nitrogen in the Leaves*

Treatments		Mean nitrogen content (% of dry matter)				Total amount of nitrogen (gm. per cut per pot)			
		Expt. I	II	III	IV	Expt. I	II	III	IV
1.0 Daylight	Control	4.20	3.85	3.60	4.46	0.045	0.077	0.106	0.138
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.18	5.07	4.73	5.32	0.125	0.201	0.247	0.262
	Ca(NO <sub>3</sub> ) <sub>2</sub>	5.05	5.09	4.80	5.37	0.119	0.198	0.225	0.224
0.63-0.61 Daylight	Control	4.26	4.18	4.22	4.92	0.048	0.087	0.123	0.136
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.20	5.13	4.97	5.52	0.076	0.158	0.173	0.179
	Ca(NO <sub>3</sub> ) <sub>2</sub>	4.95	5.11	4.80	5.27	0.076	0.136	0.146	0.127
0.44-0.37 Daylight	Control	4.03	4.18	4.48	5.02	0.040	0.078	0.104	0.114
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.12	4.99	5.00	5.39	0.064	0.099	0.110	0.102
	Ca(NO <sub>3</sub> ) <sub>2</sub>	4.73	4.66	4.77	5.19	0.066	0.079	0.096	0.082

TABLE II

*The Effects of Light Intensity on the Accumulation of Nitrogen in the Leaves of Nitrogenously Manured Plants*

(Statistical analysis based on regressions of difference from the controls in nitrogen content (% of dry matter) against the number of defoliations.)

Experiments I-IV

		Nitrogen treatments		
		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Ca(NO <sub>3</sub> ) <sub>2</sub>	Mean
Light treatments	1.0 Daylight	0.065	0.076	0.071
	0.44-0.37 Daylight	-0.006	-0.023	-0.014
	Mean	0.030	0.027	

Significant difference between treatments = 0.088

„ „ „ mean of 2 treatments = 0.062

Experiments II-IV

		Nitrogen treatments		
		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Ca(NO <sub>3</sub> ) <sub>2</sub>	Mean
Light treatments	1.0 Daylight	0.133	0.154	0.144
	0.44-0.37 Daylight	0.020	0.005	0.013
	Mean	0.077	0.080	

Significant difference between treatments = 0.027

„ „ „ means of 2 treatments = 0.013

If experiments I-IV are considered together then there is a significant difference between the effects of additional nitrogen at the two light levels. In daylight the increased nitrogen content of the leaves from manured plants rises with the number of cuts, whereas in 0.44-0.37 of daylight it tends to fall.

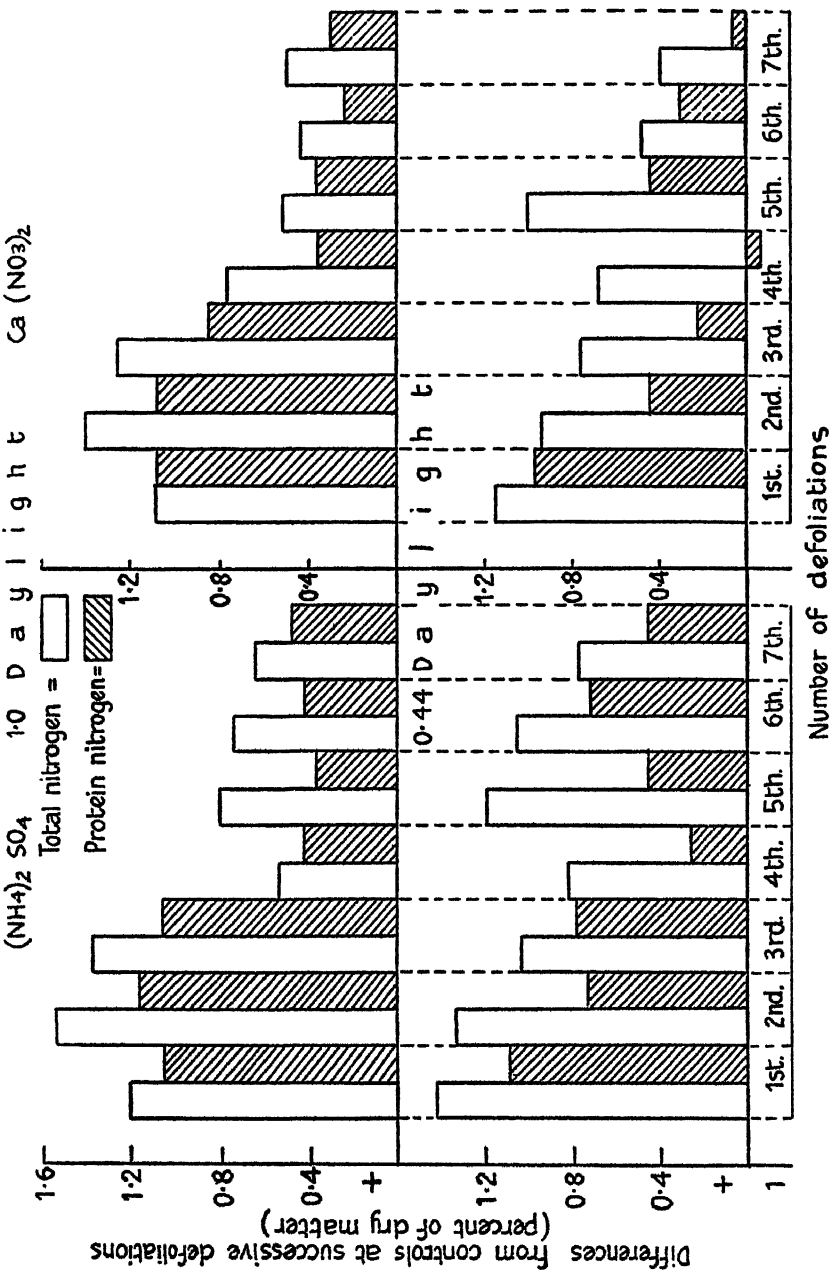


Fig. 5. Experiment I. The effects of light intensity and of additional nitrogen on the gains or losses, relative to the control changes, in the total and protein nitrogen contents of the leaves of *A. tenuis* at successive defoliations.

There is no appreciable difference in the effects of ammonium sulphate and calcium nitrate whether or not the data for experiment I are included in the analyses. Omitting experiment I does, however, lower the error and accentuate the significance of the light effects.

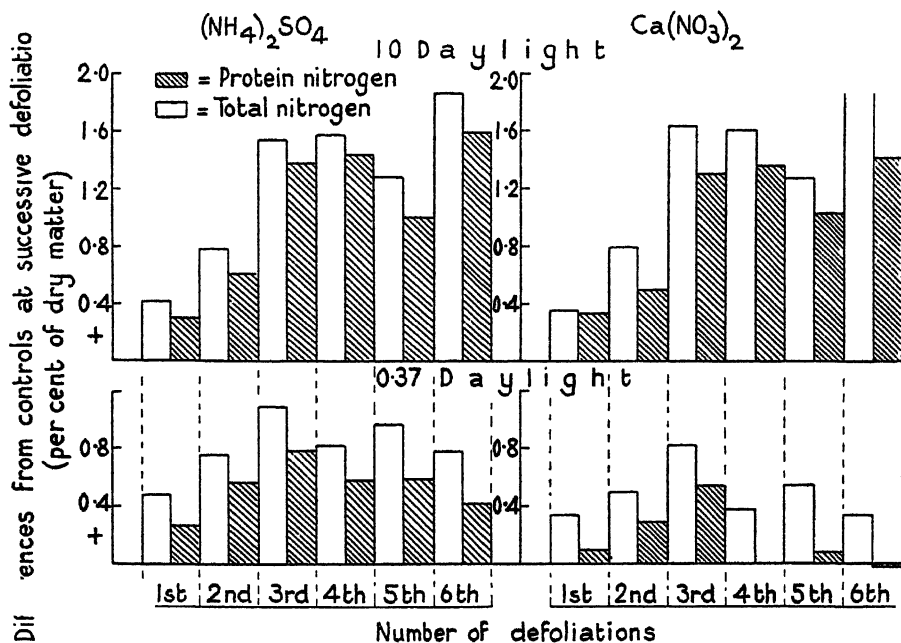


FIG. 6. Experiment II. The effects of light intensity and of additional nitrogen on the gains or losses, relative to the control changes, in the total and protein nitrogen contents of the leaves of *A. tenuis* at successive defoliations.

Apart, however, from a consideration of percentage nitrogen content there is also the question of the effect of the treatments on the absolute amount of nitrogen present in the leaves. Since this amount is dependent upon both leaf production and the percentage nitrogen content, and since the effects of nitrogen and light intensity on leaf production are more marked than on nitrogen content (compare Fig. 1 and Table I), one would expect the changes in the amount of nitrogen to follow more closely changes in leaf production. That this is so is seen in Table I. Where no nitrogen has been applied changes in the light intensity have no marked effect on the amount of nitrogen in the leaves. In daylight additional nitrogen has more than doubled the amount of nitrogen. At the level of 0.63–0.61 daylight there is again a considerable increase as a result of manuring, more particularly where ammonium sulphate is added. At the lowest light level only in experiments I and II is there any evidence that the absolute amount of nitrogen has been increased by manuring; in fact, the results for experiments III and IV point to a decrease, more especially in the case of calcium nitrate.

In experiments I–III estimates of the protein nitrogen were made on the samples collected from the plants growing either in full daylight or 0.44–0.37 of daylight, while in experiment IV determinations were also carried out at the

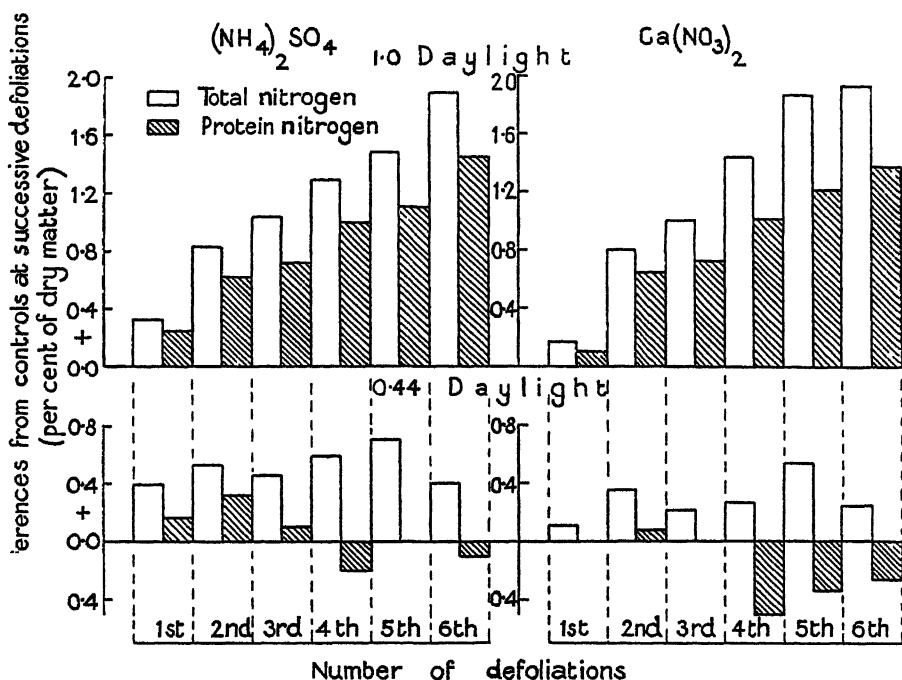


FIG. 7. Experiment III. The effects of light intensity and of additional nitrogen on the gains or losses, relative to the control changes, in the total and protein nitrogen contents of the leaves of *F. rubra* at successive defoliations.

intermediate light level. The effects of light intensity on the protein content of the leaves from the unmanured series are seen in Fig. 4 and Table III. The general trend is similar to that of the total nitrogen data. Just as lowering the light intensity increases the total nitrogen content in experiments II–IV, so a reduction in the light level raises the protein content. In experiment I the trend is again different from experiments II–IV, for in daylight the protein content is greatest.

Reference has already been made to Figs. 5–8; these, in addition to demonstrating the effects of light intensity and increased nitrogen supply on the accumulation of nitrogen in the leaves, also show their influence on the conversion of this nitrogen into protein. At the highest light intensity the results are very similar in all experiments. The increase in nitrogen content over the control as a result of the addition of either ammonium sulphate or calcium nitrate is correlated very closely with an increase in protein content. In

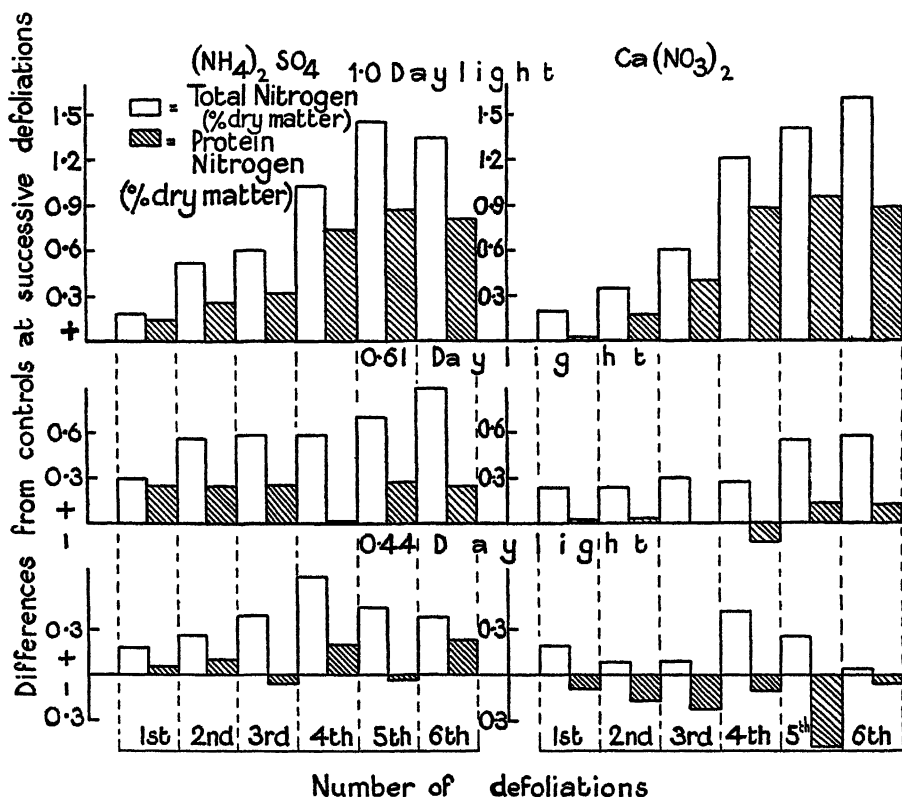


FIG. 8. Experiment IV. The effects of light intensity and of additional nitrogen on the gains or losses, relative to the control changes, in the total and protein nitrogen contents of the leaves of *A. tenuis* at successive defoliations.

TABLE III

*The Influence of Light Intensity and Nitrogen Supply on the Percentage Content and Amount of Protein Nitrogen in the Leaves*

Treatments		Mean protein nitrogen content (% of dry matter)				Total amount of protein nitrogen (gm. per cut per pot)			
		Expt. I	II	III	IV	Expt. I	II	III	IV
1.0 Daylight	Control	3.60	3.30	3.12	3.84	0.039	0.065	0.093	0.117
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4.31	4.34	3.97	4.39	0.103	0.170	0.208	0.215
	Ca(NO <sub>3</sub> ) <sub>2</sub>	4.21	4.28	3.97	4.40	0.098	0.166	0.186	0.184
0.63-0.61 Daylight	Control	—	—	—	4.10	—	—	—	0.113
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	—	—	—	4.33	—	—	—	0.140
	Ca(NO <sub>3</sub> ) <sub>2</sub>	—	—	—	4.13	—	—	—	0.102
0.44-0.37 Daylight	Control	3.43	3.52	3.69	4.16	0.034	0.066	0.085	0.094
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4.07	4.04	3.75	4.24	0.051	0.080	0.083	0.078
	Ca(NO <sub>3</sub> ) <sub>2</sub>	3.77	3.68	3.53	3.96	0.052	0.062	0.073	0.062

experiments II–IV (Figs. 6–8) as the difference in nitrogen level from the control rises with successive cuts so does the difference in protein. In experiment I (Fig. 5) where, in contrast, the difference in nitrogen content falls with time the protein nitrogen also decreases.

Although in full daylight the accumulation of nitrogen in the leaves of manured plants is coupled with an increase in protein nitrogen, at the lowest light level increases in nitrogen are not always followed by increases in protein. This difference in the effects of high and low light is most marked in experiments III and IV and least in experiment I. In experiment I under the low light intensity approximately half the increase in nitrogen can be accounted for as protein (see Tables I and III), while in daylight approximately three-quarters of the nitrogen is found as protein. In experiment II this divergence is again evident; with daylight of 75–85 per cent. of the nitrogen accumulated is present as protein, whereas in 0.43 daylight the proportion is 33–64 per cent. Although in experiments I and II the increase in nitrogen under the low light intensity is associated with a gain in protein, the results for experiments III and IV demonstrate that an increase in nitrogen may be even negatively correlated with protein level. From Fig. 7 and Table III it is seen that on average the increase in nitrogen content over the control cannot in experiment III be accounted for as protein. On the contrary, especially where calcium nitrate is added, the rise in nitrogen is coupled with a loss of protein. In experiment IV (Fig. 8) there is a marked gain in protein in full daylight. At the intermediate light level there is some increase in protein with the ammonium sulphate treatment, and on average no change in the case of calcium nitrate. Under the lowest light intensity the plants manured with ammonium sulphate have gained a little protein but less than at the intermediate light level, while as a result of the application of calcium nitrate there has been a loss of protein in the leaves.

Besides the divergent effects of high and low light intensity on the balance between total nitrogen and protein, the influence of the frequency of defoliation must be taken into account. Under high light, from the initial to the final cut, there is a gradual increase in the protein content of manured plants over that of the controls except in experiment I (Fig. 5). But with the lowest light intensity in all four experiments there is a fall in the protein difference with time (Figs. 5–8). That these two trends under the two light levels are significantly different is seen in Table IV, whether the data for experiment I are included or not in the statistical analysis. On the other hand, neither in high or low light are the regressions for ammonium sulphate or calcium nitrate dissimilar.

The results for the total amount of protein nitrogen are set out in Table III. From the data it is seen that on this basis the effects of light intensity and nitrogen supply are even more accentuated than on the basis of percentage dry matter. In full daylight additional nitrogen has led to a very marked rise in protein nitrogen. At lower light levels there is a very considerable drop in

protein accumulation as a result of manuring. In fact, although in experiment I an increase in protein follows on the application of either ammonium sulphate or calcium nitrate, yet in experiment IV an increase in the nitrogen supply leads to a decrease in protein.

TABLE IV

*The Effects of Light Intensity on the Accumulation of Protein Nitrogen in the Leaves of Nitrogenously Manured Plants*

(Statistical analyses based on regressions of difference from controls in protein nitrogen content (% of dry matter) against number of cuts.)

Experiments I-IV		Nitrogen treatments		
		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Ca(NO <sub>3</sub> ) <sub>2</sub>	Mean.
Light treatments	1.0 Daylight	0.040	0.041	0.040
	0.44-0.37 Daylight	-0.025	-0.044	-0.035
	Mean	0.007	-0.002	

Significant differences between treatments = 0.088

„ „ „ means of 2 treatments = 0.062

Experiments II-IV		Nitrogen treatments		
		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Ca(NO <sub>3</sub> ) <sub>2</sub>	Mean.
Light treatments	1.0 Daylight	0.100	0.108	0.104
	0.44-0.37 Daylight	-0.007	-0.026	-0.016
	Mean	0.047	0.041	

Significant difference between treatments = 0.042

„ „ „ mean of 2 treatments = 0.029

*Non-protein nitrogen level in relation to nitrogen supply and light intensity.*

In order to interpret more fully the changes in the non-protein nitrogen, this fraction was further analysed into nitrate nitrogen and 'organic' non-protein nitrogen. The term 'organic', which is used for the sake of brevity, is a slight misnomer as it includes any ammonia nitrogen present in the leaves. However, the proportions of ammonia nitrogen found in a number of samples (see Table IX) are extremely small, and thus this term is likely to be misleading only to a very small extent.

*Nitrate nitrogen.* The influence of light intensity and nitrogen supply on the nitrate nitrogen content of the leaves is even more marked than on the total nitrogen and protein levels. The effect of varying light intensity in the absence of additional nitrogen is seen in Fig. 9 and Table V. In all experiments the leaves under the low light intensity contain more nitrate nitrogen, particularly in experiments II-IV. In full daylight the addition of both ammonium sulphate and calcium nitrate has greatly increased the nitrate nitrogen level over that of the control, more so in fact than at the lower light levels (see Table V).

The relationship between the accumulation of total and nitrate nitrogen in the leaves of manured plants is seen in Figs. 9-12. As in the case of the



TABLE V

*The Influence of Light Intensity and Nitrogen Supply on the Content and Amount of Nitrate Nitrogen in the Leaves*

Treatments		Mean nitrate nitrogen content (% of dry matter)				'Total amount of nitrate nitrogen (gm. per cut per pot)			
		Expt. I	II	III	IV	Expt. I	II	III	IV
Daylight	Control	0.051	0.027	0.081	0.109	0.0006	0.001	0.002	0.003
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.219	0.087	0.292	0.229	0.0057	0.004	0.012	0.014
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.217	0.144	0.274	0.314	0.0053	0.006	0.012	0.013
0.63-0.61 Daylight	Control	—	—	—	0.199	—	—	—	0.006
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	—	—	—	0.456	—	—	—	0.015
	Ca(NO <sub>3</sub> ) <sub>2</sub>	—	—	—	0.505	—	—	—	0.012
0.44-0.37 Daylight	Control	0.071	0.124	0.293	0.352	0.0006	0.002	0.007	0.008
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.331	0.269	0.598	0.622	0.0041	0.005	0.012	0.011
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.402	0.346	0.696	0.711	0.0053	0.005	0.012	0.011

protein accumulation the divergent effects of high and low light intensities are most marked in experiments III and IV (Figs. 11 and 12) and least in experiment I (Fig. 9). In daylight the increase in total nitrogen content is accompanied by a relatively small increase in nitrate nitrogen. The average proportion that the nitrate nitrogen increase bears to the total nitrogen rise ranges from 5 to 10 per cent. for the ammonium sulphate and calcium nitrate treatments in experiment II to 22 per cent. for both nitrogen treatments in experiment IV. But at a light intensity of 0.44-0.37 of daylight the proportions are much larger; in experiment I it is 23 per cent. and 42 per cent. after the addition of ammonium sulphate and calcium nitrate respectively, and in experiment IV 73 per cent. and 210 per cent. Moreover, at the lower light levels the accumulation of nitrate nitrogen becomes in each experiment progressively greater with successive defoliations; thus the ratio of nitrate to total nitrogen rises steadily with time. This is especially evident in Figs. 11 and 12 where, more particularly with calcium nitrate, the nitrate nitrogen increase may in the final cuts be more than four times the increase in total nitrogen. With full daylight there is also a tendency for the nitrate nitrogen to rise, but except in experiment I this is linked with an increase in the total nitrogen. From Table VI it is seen that for experiments I-IV this trend for the nitrate nitrogen to accumulate with repeated cutting is not significantly affected either by the source of the nitrogen or the light intensity. If experiment I is, however, omitted the mean light effect only just fails to reach a level of significance (see lower half of table).

The absolute amounts of nitrate nitrogen present in the leaves are in line with the results on a basis of dry matter (see Table V). In daylight, the nitrate nitrogen following manuring rises at least to four times and may reach nine times that in the corresponding control. At the lower light intensities this rise, except in experiment I, is less than in full daylight.

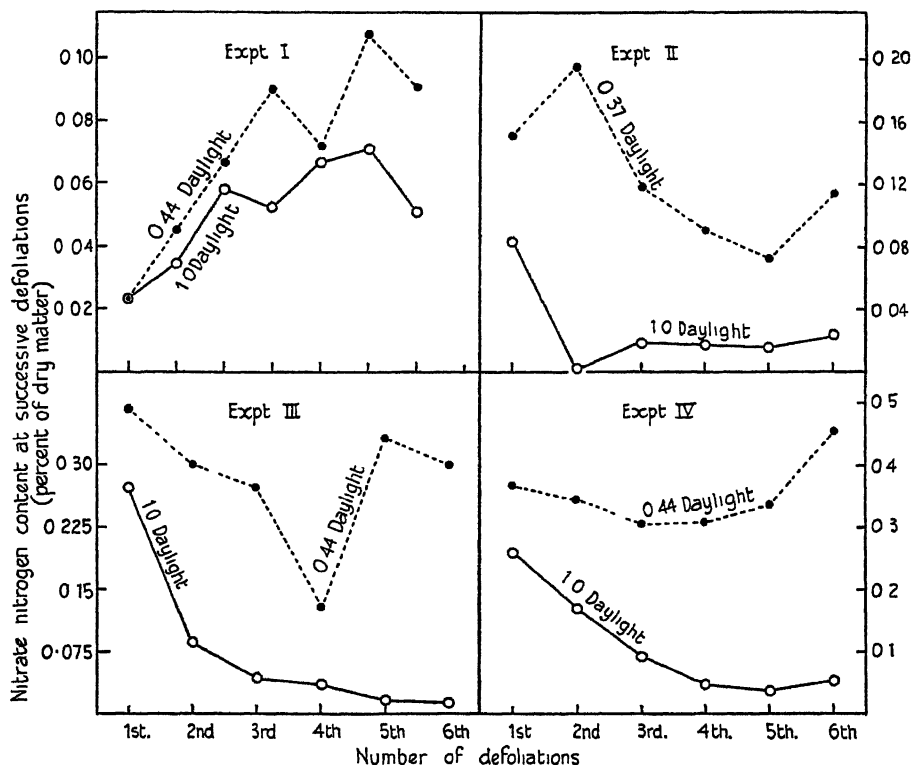


FIG. 9. The effects of light intensity on the nitrate nitrogen content of the leaves of control plants (low nitrogen supply) at successive defoliations. (Experiments I, II, and IV *A. tenuis*; experiment III *F. rubra*.)

TABLE VI

*The Effects of Light Intensity on the Accumulation of Nitrate Nitrogen in the Leaves of Nitrogenously Manured Plants*

(Statistical analyses based on regressions of difference from the controls in nitrate nitrogen content (% of dry matter) against number of cuts.)

Experiments I-IV

Light treatments	Nitrogen treatments		Mean.
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Ca(NO <sub>3</sub> ) <sub>2</sub>	
1.0 Daylight	0.011	0.014	0.013
0.43-0.37 Daylight	0.013	0.025	0.019
Mean	0.012	0.020	

Significant difference between means = 0.021

" " " means of 2 treatments = 0.015

Experiments II-IV

Light treatments	Nitrogen treatments		Mean.
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Ca(NO <sub>3</sub> ) <sub>2</sub>	
1.0 Daylight	0.010	0.012	0.011
0.44-0.37 Daylight	0.022	0.028	0.025
Mean	0.016	0.020	

Significant difference between treatments = 0.021

" " " means of 2 treatments = 0.015

'Organic' non-protein nitrogen. Reference to Fig. 13 and Table VII shows that the effects of shading on the 'organic' non-protein nitrogen content in the control leaves. Except in experiment III the 'organic' nitrogen content in full daylight and in 0.44–0.37 of daylight is on average substantially the same. In full daylight the general trends of the changes with successive cuts follow those in the protein and nitrate nitrogen contents. At the lower light intensity the fall with time in 'organic' non-protein nitrogen in experiments II–IV is more marked than in the case of the nitrate nitrogen changes.

TABLE VII

*The Influence of Light Intensity and Nitrogen Supply on the Content and Amount of 'Organic' Non-protein Nitrogen in the Leaves*

Treatments		Mean 'organic' non-protein nitrogen content (% of dry matter)				Total amount of 'organic' non-protein nitrogen (gm. per cut per pot)			
		Expt. I	II	III	IV	Expt. I	II	III	IV
Daylight	Control	0.554	0.517	0.395	0.513	0.006	0.011	0.011	0.018
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.649	0.643	0.532	0.645	0.015	0.027	0.026	0.033
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.629	0.662	0.558	0.650	0.015	0.026	0.027	0.027
0.63 Daylight	Control	—	—	—	0.617	—	—	—	0.018
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	—	—	—	0.737	—	—	—	0.023
	Ca(NO <sub>3</sub> ) <sub>2</sub>	—	—	—	0.640	—	—	—	0.015
0.44–0.37 Daylight	Control	0.533	0.542	0.498	0.505	0.005	0.010	0.012	0.012
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.720	0.672	0.653	0.533	0.009	0.014	0.015	0.013
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.564	0.635	0.553	0.515	0.008	0.012	0.011	0.009

At all light intensities the addition of either ammonium sulphate and calcium nitrate raises the 'organic' non-protein nitrogen content. In full daylight the mean increases are of the same order in the several experiments, whereas at the lowest light intensity the increases are more marked in experiments I–III than they are in experiment IV (see Table VII). This accumulation of 'organic' non-protein nitrogen is also dependent upon the number of defoliations. The increases in 'organic' nitrogen as a result of manuring tend in full daylight to rise with successive defoliations especially in experiments II–IV (Figs. 10–12). At 0.43–0.37 of daylight there is little evidence of such a rise. That this difference in trend either for the mean of the four or three experiments is in fact significant is seen in Table VIII. Both the effects of calcium nitrate and the mean effects of additional nitrogen are significantly different under high and low light intensity.

No attempt was made in every sample to separate the 'organic' non-protein nitrogen into further fractions on account of the labour and time involved. However, such separations were carried out on material from experiments III and IV and the results of these analyses are given in Table IX.

Considering first the amino nitrogen data, it is seen that in experiment III a reduction in the light intensity has little effect on this fraction in the absence of nitrogenous manuring, while in experiment IV the amino nitrogen

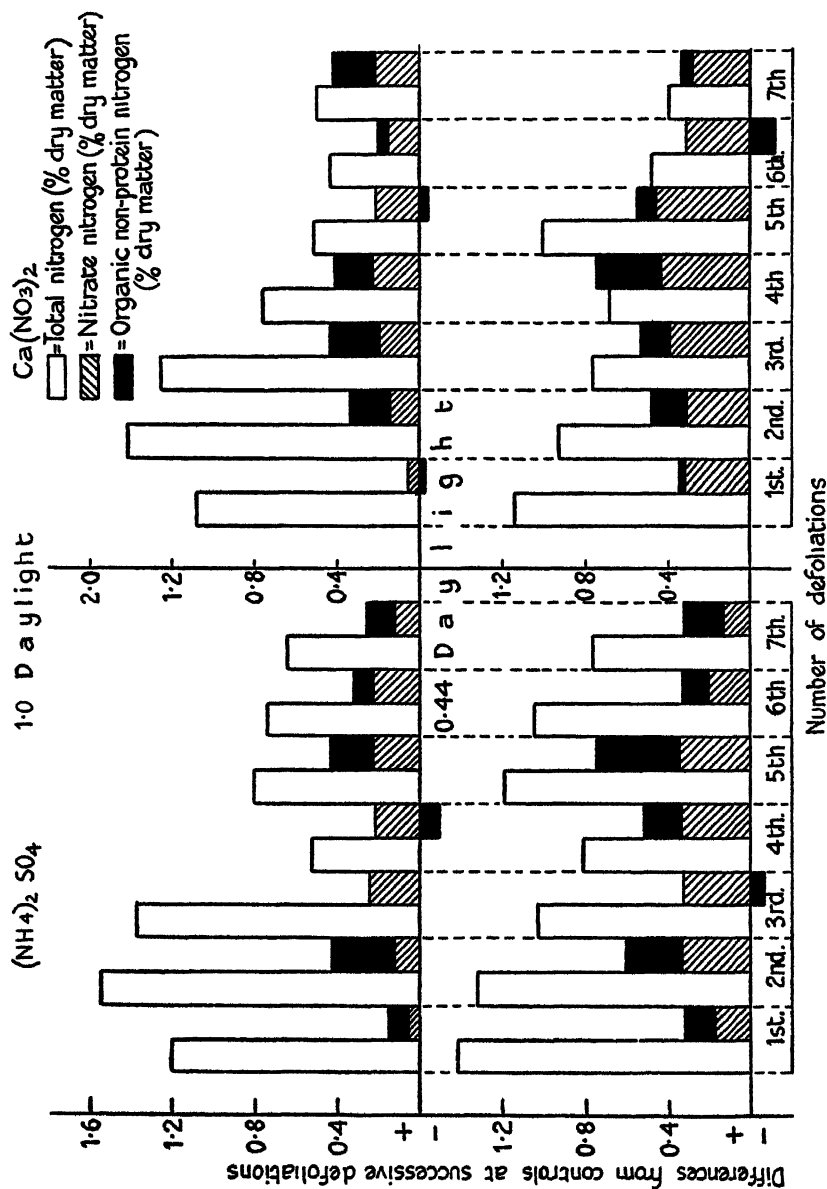


FIG. 10. Experiment I. The effects of light intensity and of additional nitrogen on the gains or losses, relative to the control changes, in the total nitrate and non-protein organic nitrogen contents of the leaves of *A. tenuis* at successive defoliations.

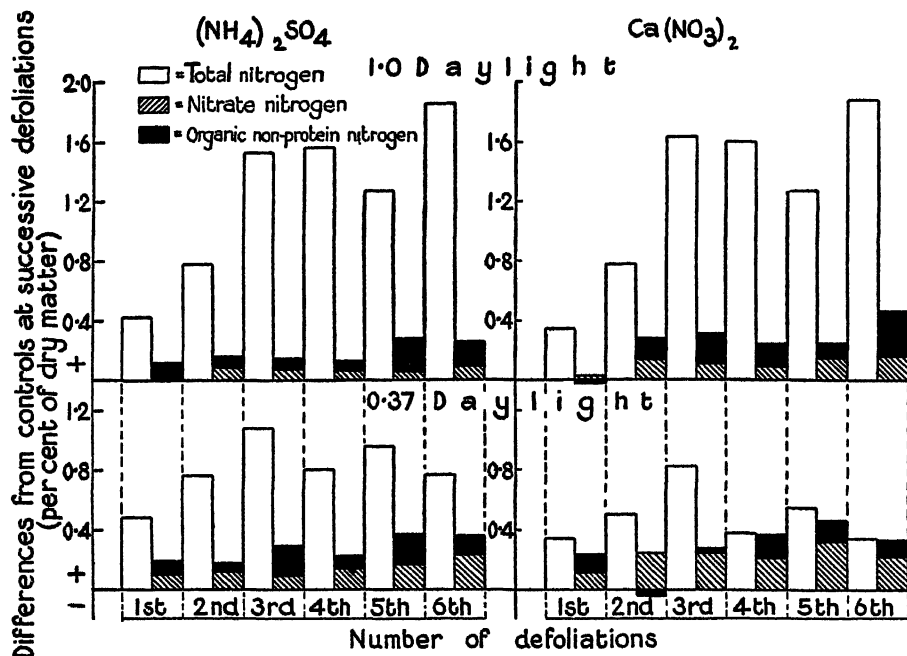


FIG. 11. Experiment II. The effects of light intensity and of additional nitrogen on the gains or losses, relative to the control changes, in the total nitrate and non-protein organic nitrogen contents of the leaves of *A. tenuis* at successive defoliations.

TABLE VIII

*The Effects of Light Intensity on the Accumulation of 'Organic' Non-protein Nitrogen in the Leaves of Nitrogenously Manured Plants*

(Statistical analyses based on regressions of difference from the controls in 'organic' non-protein nitrogen content (% of dry matter) against number of cuts.)

Experiments I-IV		Nitrogen treatments		
		$(\text{NH}_4)_2\text{SO}_4$	$\text{Ca}(\text{NO}_3)_2$	Mean
Light treatments	1.0 Daylight	0.016	0.028	0.022
	0.44-0.37 Daylight	0.005	-0.009	-0.002
	Mean	0.011	0.009	
Significant difference between treatments = 0.021				
,, ,, ,,		means of 2 treatments = 0.015		
Experiments II-IV		Nitrogen treatments		
		$(\text{NH}_4)_2\text{SO}_4$	$\text{Ca}(\text{NO}_3)_2$	Mean
Light treatments	1.0 Daylight	0.023	0.034	0.029
	0.44-0.37 Daylight	0.003	-0.006	-0.001
	Mean	0.013	0.014	
Significant difference between treatments = 0.026				
,, ,, ,,		means of 2 treatments = 0.018		

level is perhaps higher at the lower light intensity. In full daylight the addition of both ammonium sulphate and calcium nitrate has increased to some small extent the amino nitrogen. But at the level of 0.44-0.37 of daylight, although initially ammonium sulphate and to a less extent calcium nitrate may have raised the amino nitrogen content, yet in the final cut the manured leaves contain rather less than the control.

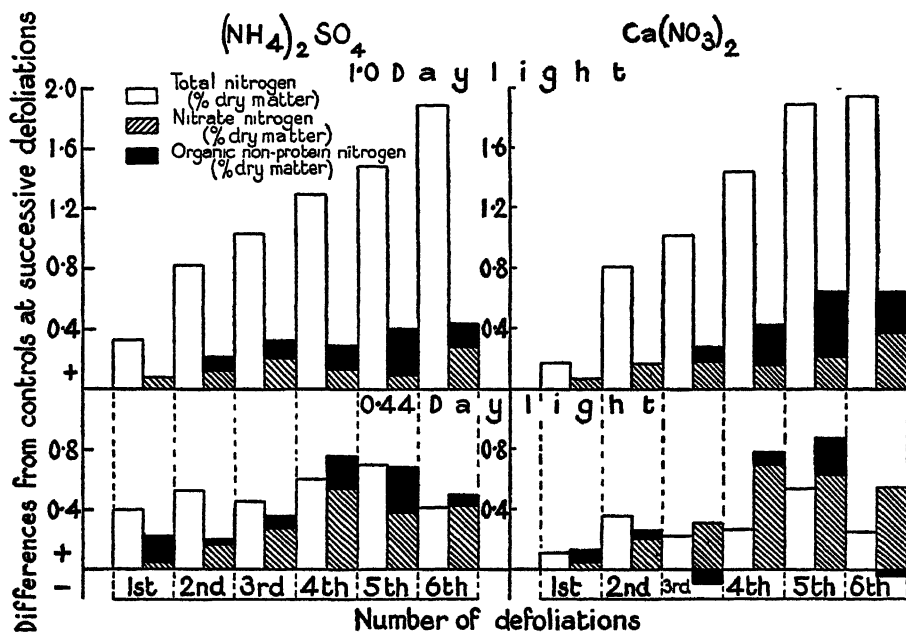


FIG. 12. Experiment III. The effects of light intensity and of additional nitrogen on the gains or losses, relative to the control changes, in the total nitrate and non-protein organic nitrogen contents of the leaves of *F. rubra*.

The data for amide nitrogen show that changes in light intensity and nitrogen supply have only minor effects. In the control plants lowering the light intensity causes either no change in the amide nitrogen level or increases it slightly. Similarly, the addition of nitrogen at either light intensity brings about no consistent result. Save for experiment III, where manuring has increased considerably the amide nitrogen content of the shaded leaves, the amide fraction relative to the control rises and falls irregularly.

In the majority of samples the unidentified organic non-protein nitrogen or 'rest' nitrogen equals or exceeds the amino plus amide nitrogen. From Table IX it is seen that with the unmanured plants a reduction in the light intensity in experiment III increases the 'rest' fraction, but in experiment IV this trend is reversed. The addition of either ammonium sulphate or calcium nitrate in full daylight, particularly in experiment III, brings about a rise, whereas at 0.44-0.37 of daylight additional nitrogen depresses the 'rest' nitrogen.

TABLE IX  
*Influence of Light Intensity and Nitrogen Supply on Non-protein Nitrogen of Leaves.*  
*Non-protein Nitrogen Fractions (percentage of dry matter)*

Experiment III. <i>Festuca rubra</i>																					
Amino nitrogen.						Amide nitrogen.				'Rest' nitrogen.				Ammonia nitrogen.				Nitrate nitrogen.			
Cut		2	4	6	Mean	2		4	6	Mean	2		4	6	Mean	2		4	6	Mean	
Day-light	Control	0.037	0.098	0.132	0.089	0.056	0.040	0.013	0.036	0.087	0.199	0.205	0.164	0.027	0.023	0.040	0.030	0.087	0.037	0.011	0.045
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.050	0.100	0.135	0.095	0.084	0.042	0.134	0.087	0.206	0.362	0.269	0.279	0.013	0.029	0.051	0.031	0.206	0.167	0.284	0.219
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.069	0.070	0.162	0.100	0.051	0.051	0.062	0.035	0.251	0.477	0.354	0.361	0.009	0.026	0.035	0.023	0.251	0.199	0.378	0.276
Day-light	Control	0.098	0.098	0.115	0.104	0.071	0.041	0.037	0.050	0.364	0.214	0.259	0.279	0.018	0.017	0.035	0.023	0.314	0.135	0.307	0.252
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.133	0.103	0.128	0.121	0.054	0.112	0.139	0.102	0.348	0.329	0.225	0.301	0.038	0.065	0.042	0.048	0.487	0.679	0.717	0.628
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.075	0.092	0.094	0.087	0.114	0.097	0.143	0.118	0.389	0.206	0.133	0.243	0.033	0.035	0.030	0.033	0.514	0.825	0.852	0.730
Experiment IV. <i>Agrostis tenuis</i>																					
Cut		2	4	6	Mean	2		4	6	Mean	2		4	6	Mean	2		4	6	Mean	
Day-light	Control	0.072	0.053	0.075	0.067	0.079	0.064	0.095	0.079	0.345	0.326	0.244	0.305	0.003	0.012	0.004	0.006	0.167	0.048	0.052	0.089
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.084	0.095	0.139	0.106	0.088	0.064	0.130	0.094	0.359	0.383	0.368	0.370	0.002	0.012	0.012	0.009	0.326	0.218	0.356	0.300
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.110	0.093	0.121	0.108	0.070	0.099	0.073	0.081	0.315	0.309	0.351	0.325	0.003	0.005	0.004	0.004	0.351	0.321	0.253	0.308
Day-light	Control	0.074	0.090	0.116	0.093	0.079	0.083	0.073	0.078	0.261	0.141	0.139	0.264	0.003	0.003	0.012	0.006	0.345	0.308	0.453	0.369
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.096	0.115	0.102	0.104	0.096	0.080	0.097	0.089	0.141	0.109	0.298	0.183	0.005	0.019	0.012	0.012	0.576	0.741	0.677	0.665
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.103	0.102	0.110	0.105	0.069	0.075	0.081	0.075	0.202	0.073	0.310	0.195	0.004	0.019	0.012	0.011	0.049	0.885	0.639	0.724

There finally remains the ammonia nitrogen which in none of the samples is found in any appreciable amount. Furthermore, neither alterations in the light intensity nor the nitrogen level have resulted in marked changes. In the controls the ammonia content is much the same at both light intensities. The addition of ammonium sulphate has in full daylight only raised the

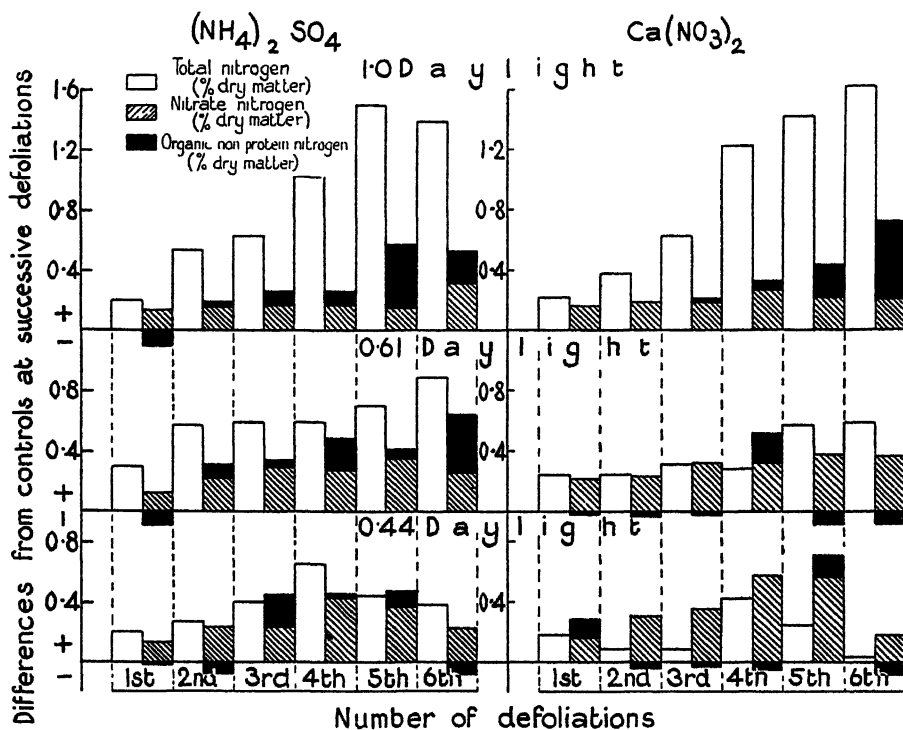


FIG. 13. Experiment IV. The effects of light intensity and of additional nitrogen on the gains or losses, relative to the control changes, in the total nitrate and non-protein organic nitrogen contents of the leaves of *A. tenuis* at successive defoliations.

ammonia content to a slight extent, while even at the lower light intensity there is no pronounced rise as might be expected if the plants were suffering from 'ammonia' poisoning.

Considering the 'organic' non-protein nitrogen data in terms of absolute amount rather than on a percentage basis much the same conclusions are reached. In manured plants the 'organic' non-protein nitrogen level is similar at the different light intensities (see Table VII). In daylight additional nitrogen very considerably increases the 'organic' non-protein level in all experiments. At the lowest light intensity ammonium sulphate, except in experiment IV, has raised the 'organic' fraction to a small extent. Calcium nitrate has only brought about a comparable rise in experiment I, while in experiment IV the 'organic' non-protein nitrogen is depressed.



In addition to a study of the nitrogen changes the effects of the experimental conditions on the water-soluble carbohydrates were also investigated. No attempt has been made for each sample to separate the carbohydrates into sucrose reducing sugars and other components. Analysis of material mainly

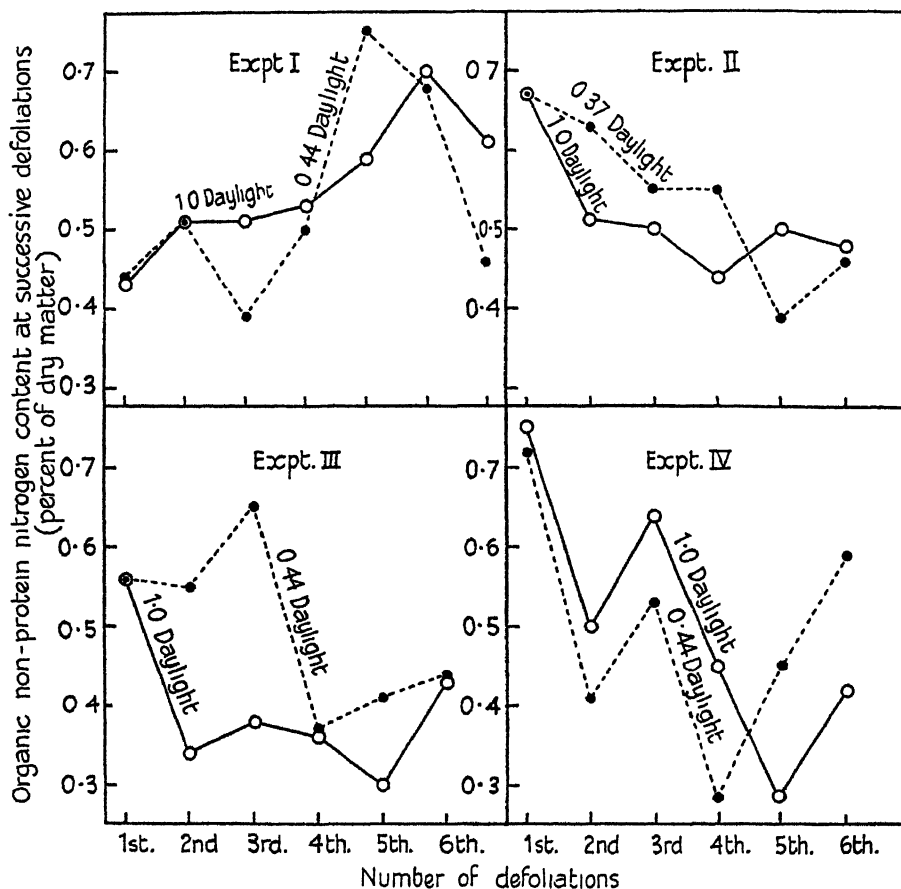


FIG. 14. The effects of light intensity on the non-protein organic nitrogen content of the leaves of control plants (low nitrogen supply) at successive defoliations. (Experiments I, II, and IV, *A. temis*; experiment III, *F. rubra*.)

from experiments III and IV has shown that the bulk of the water-soluble carbohydrates consists principally of sucrose and reducing sugars and that fructosan is only present in small amounts (Blackman and Templeman, 1939). Some thirty-six additional analyses have demonstrated that the reducing sugar content of the water extract after inversion with invertase is on average only slightly less than that obtained with acid inversion (0.2 normal acid). For the leaves grown in full daylight invertase inversion accounts in both

experiments III and IV for 92.6 per cent. of the carbohydrates inverted with acid, while the corresponding figures for 0.44 of daylight are 86.5 and 83.8 per cent.

The changes in the water-soluble carbohydrate content of the leaves during the course of each experiment are shown in Figs. 13-16. While some of the trends are common to the four experiments others are divergent. A

TABLE X

*The Influence of Light Intensity and Nitrogen Supply on the Content and Amount of 'Water-soluble Carbohydrates' in the Leaves*

Treatments		Mean water-soluble carbohydrates as reducing sugars (% of dry matter)				Total amount of water-soluble carbohydrates (gm. per cut per pot)			
		Expt. I	II	III	IV	Expt. I	II	III	IV
Daylight	Control	8.21	6.67	6.39	6.50	0.088	0.128	0.193	0.213
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	6.31	6.41	5.69	5.91	0.148	0.238	0.297	0.315
	Ca(NO <sub>3</sub> ) <sub>2</sub>	6.78	6.67	5.30	5.74	0.152	0.250	0.262	0.257
0.63 of Daylight	Control	—	—	—	4.66	—	—	—	0.130
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	—	—	—	4.75	—	—	—	0.152
	Ca(NO <sub>3</sub> ) <sub>2</sub>	—	—	—	4.61	—	—	—	0.110
0.43-0.37 of Daylight	Control	7.02	4.95	2.61	3.06	0.068	0.093	0.064	0.071
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	6.64	5.11	2.70	3.15	0.077	0.105	0.063	0.062
	Ca(NO <sub>3</sub> ) <sub>2</sub>	6.20	5.00	2.61	3.14	0.080	0.087	0.058	0.052

reduction in the light level has a marked effect on the carbohydrate concentration. This is most clearly shown in experiments III and IV (Figs. 15 and 16, and Table X), while the smallest changes occur in experiment I (Fig. 13). In each experiment, more particularly in full daylight, the carbohydrate content fluctuates considerably between cuts. As a result, the difference in carbohydrate at the various light levels is by no means constant. For example, in experiment II (Fig. 14) the effect of light intensity is most marked in the first half of the experiment, while in experiment I (Fig. 13) this does not hold. In experiments III and IV (Figs. 15 and 16) the variation in the carbohydrate changes over the experimental period show a very close agreement. At the time of the first cut the leaves at the lowest light level contain the most carbohydrates and those exposed to full daylight the least, whereas at the time of the second and subsequent defoliations the position in both experiments is reversed.

While light intensity is the most important factor controlling the carbohydrate level, there is, nevertheless, a nitrogen effect. In full daylight, as a result either of the addition of ammonium sulphate or of calcium nitrate, there is on average a fall in content, although this is not marked in experiment II. But at the light level of 0.44-0.37 of daylight there is only evidence in experiment I that additional nitrogen causes a decrease in carbohydrates. That over the four experiments these divergent results at the two light levels are significantly different is seen in Table XI.

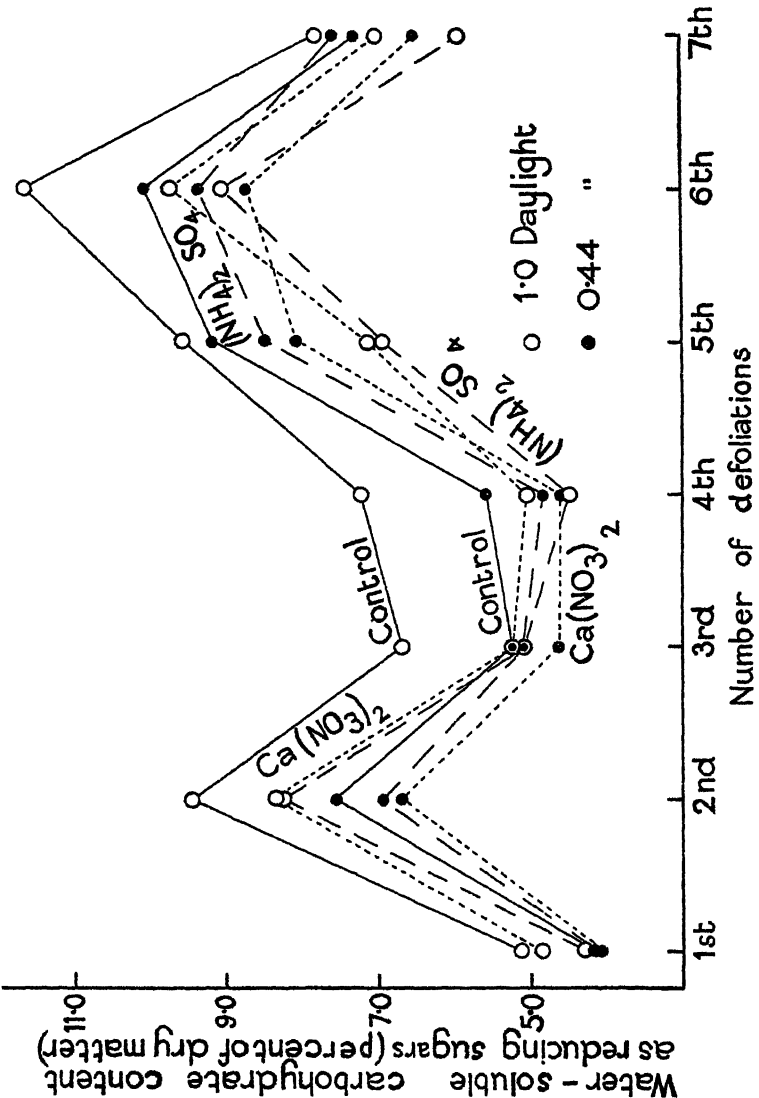


FIG. 15. Experiment I. The effects of light intensity and of nitrogen supply on the total water-soluble carbohydrate content of the leaves of *A. tenuis* at successive defoliations.

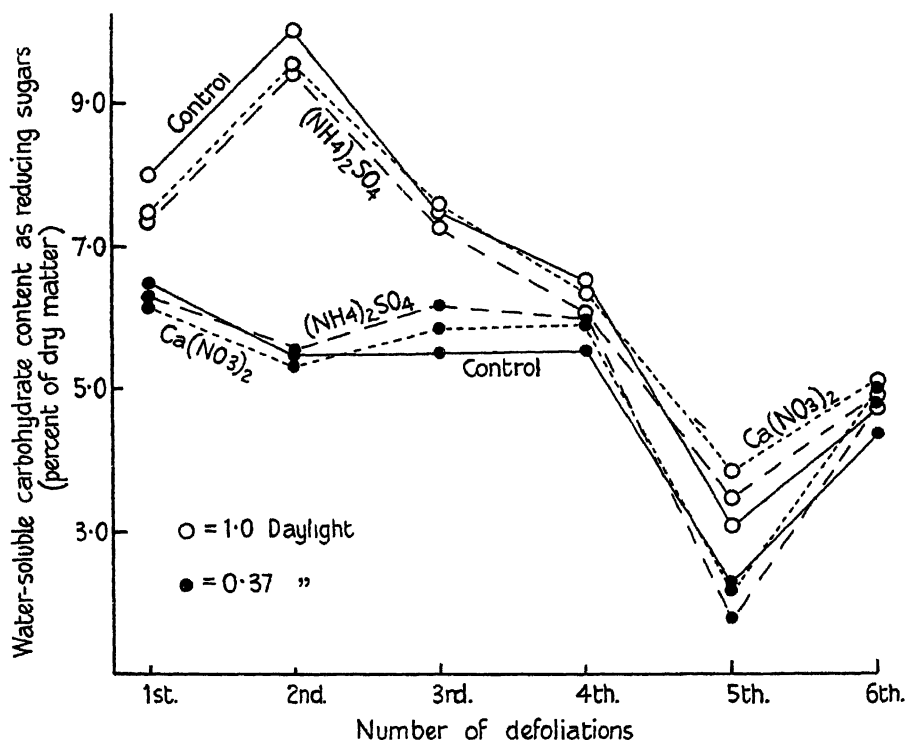


FIG. 16. Experiment II. The effects of light intensity and of nitrogen supply on the total water-soluble carbohydrate content of the leaves of *A. tenuis* at successive defoliations.

TABLE XI

*The Interaction of Light Intensity and Nitrogen Supply on the Water-soluble Carbohydrate Contents of Manured Plants Relative to the Controls*

Differences from controls of carbohydrates  
(reducing sugars as per cent. of dry matter)

		Manurial treatments		
		$(\text{NH}_4)_2\text{SO}_4$	$\text{Ca}(\text{NO}_3)_2$	Mean.
Light treatments	1.0 Daylight	-0.86	-0.82	-0.84
	0.44-0.37 Daylight	-0.01	-0.17	-0.09
	Mean	-0.44	-0.50	

Significant difference between treatments = 0.44

" " " means of 2 treatments = 0.31

As within each light intensity the nitrogen effects on the carbohydrate content are not pronounced, the total amount of carbohydrate in the leaves is largely dependent on the relative leaf production. From Table X it is seen that in full daylight additional nitrogen increases the quantity of sugars in each experiment, whereas at the lowest light level only the data for experiment I

show an increase. In each experiment irrespective of the manurial treatments the production of carbohydrates is considerably greater in full daylight.

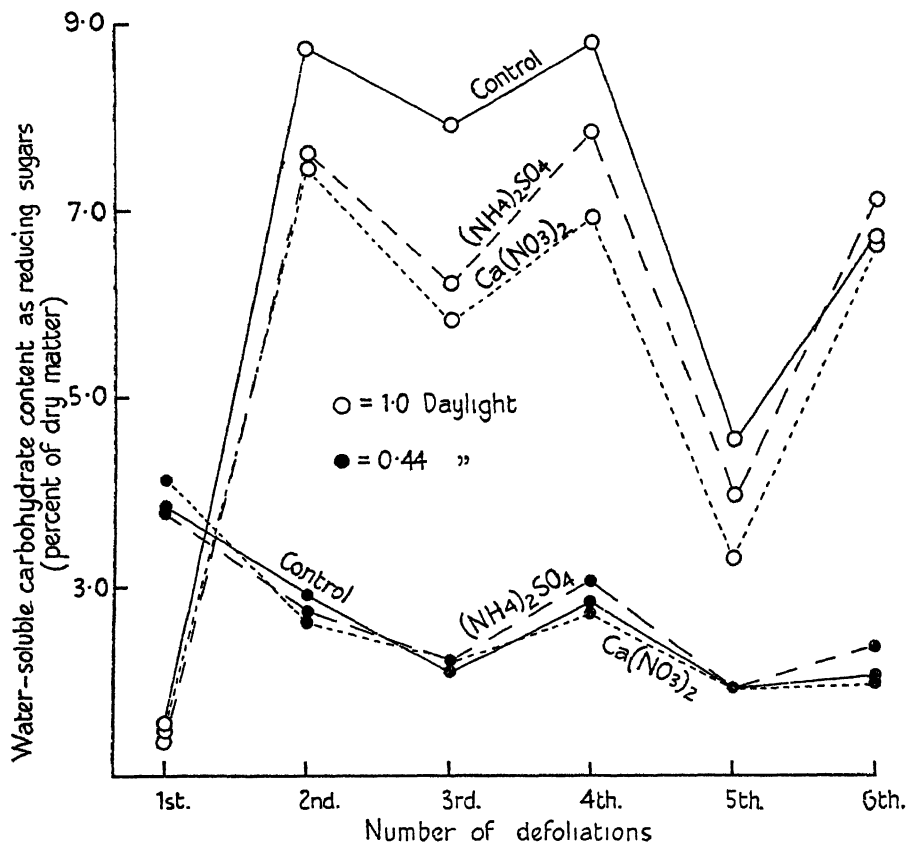


FIG. 17. Experiment III. The effects of light intensity and of nitrogen supply on the total water-soluble carbohydrate content of the leaves of *F. rubra* at successive defoliations.

*Total organic acid level in relation to nitrogen supply and light intensity.*

Although it was clear from the investigations of Vickery *et al.* (1935, 1937) that the organic acids might play an important part in the nitrogen metabolism of the majority of plants, their methods of analysis did not seem applicable to samples as small as those available in the present experiments. It was only when the original analytical programme had been completed in the summer of 1939 that the estimation of the organic acid content of the leaves became possible. By this time a micro method for the estimation of the total organic acid content had been tentatively worked out in the Biochemistry Department of the Imperial College.<sup>1</sup> Although the correction factor for the phosphates

<sup>1</sup> The method of analysis is outlined in Chibnall's recent book (1939, p. 207). A fuller and more detailed account will be published elsewhere.

present in the leaves required confirmation, it was decided, in view of the uncertain European situation, to carry out the analyses. Unfortunately, these data are not as complete as those of previous estimations, for, firstly, the work was in part interrupted by the outbreak of war and, secondly, with some

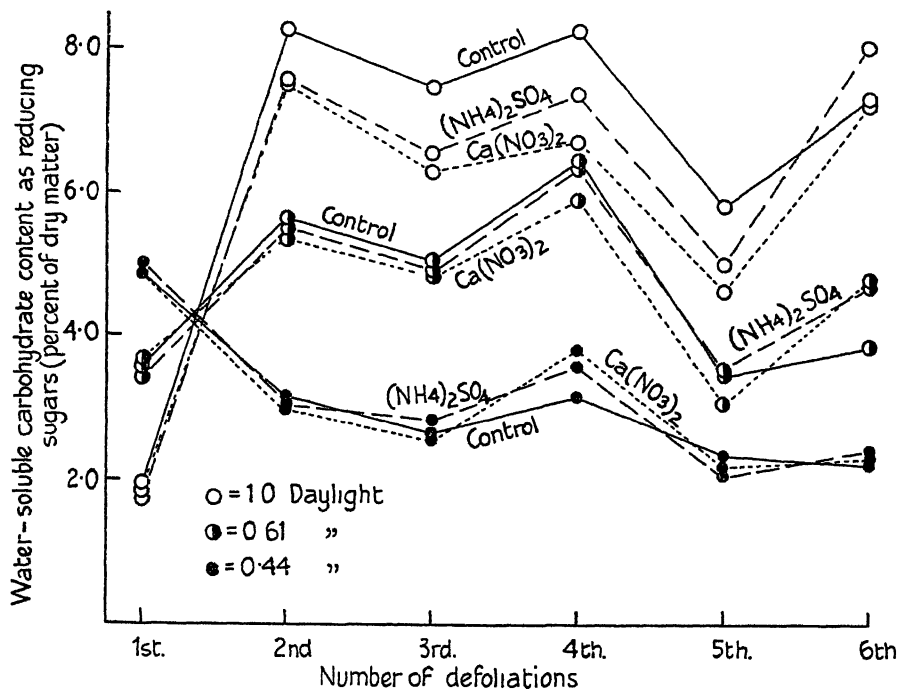


FIG. 18. Experiment IV. The effects of light intensity and of nitrogen supply on the total water-soluble carbohydrate content of the leaves of *A. tenuis* at successive defoliations.

samples there was not enough material left for analysis. Nevertheless, sufficient estimations were carried out to show the general effects in each experiment. The data are given in Figs. 19–22, where for convenience the contents have been expressed as if the only acid present was oxalacetic.

Although the evidence, more particularly in experiments II–IV, points to a reduction in the carbohydrate level as a result of shading, yet in only experiment II (Fig. 20) has shading, within each nitrogen treatment, decreased the total organic acid content to a marked extent. In experiment I (Fig. 19) there is some indication that in cuts III and V a diminution in the light intensity causes a fall in the organic acid level. However, in experiment IV (Fig. 22), where decreasing the light intensity brought about a progressive reduction in the carbohydrate level (Fig. 18), the total organic acid content within each manurial treatment remains the same at the three light intensities. Finally, in experiment III (Fig. 21) although initially the shaded leaves contained less organic acids than the unshaded, yet subsequently their content was greater.

Apart, however, from the influence of light intensity on the organic acids there is also a nitrogen effect; this is most pronounced in experiment IV (Fig. 22). At all light intensities the plants manured with calcium nitrate contain the highest and the controls the lowest content of organic acids. This gain in concentration resulting from the addition of calcium nitrate is also

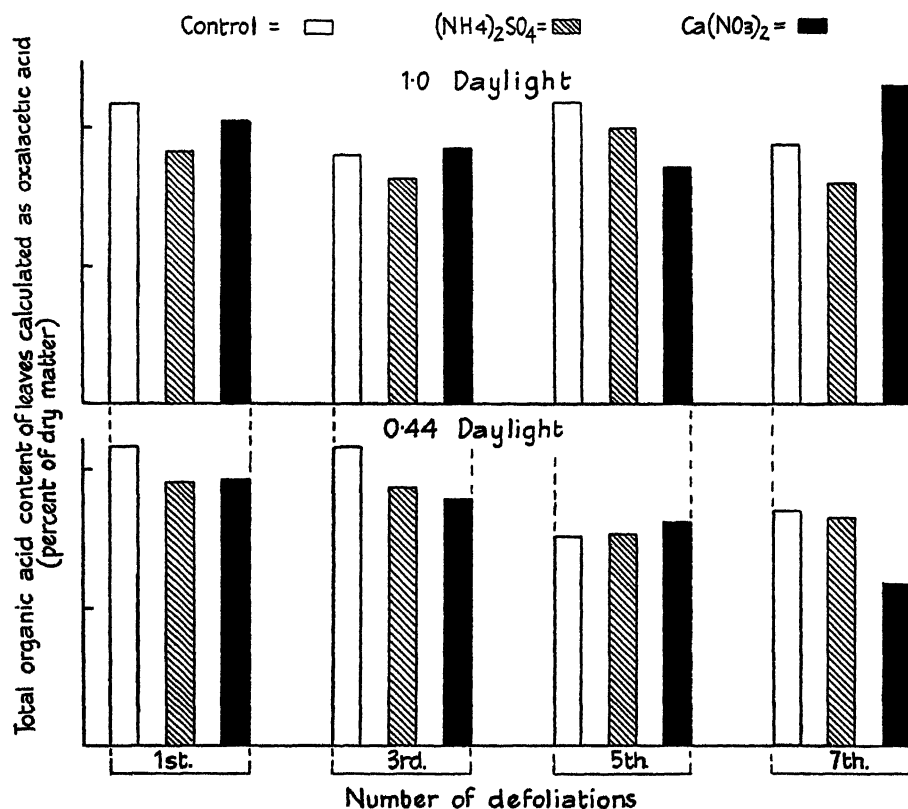


FIG. 19. Experiment I. The effects of light intensity and of nitrogen supply on the total organic acid content of the leaves of *A. tenuis* at successive defoliations.

seen in experiments II and III (Figs. 20 and 21), but in experiment I (Fig. 19) this effect is not so evident. The results for ammonium sulphate are less consistent than those for calcium nitrate. Whereas in experiment IV manuring on the average increases the acid content, yet in experiment I there is a decrease. Moreover, in experiment III there is an apparent interaction between light intensity and manuring. In daylight the mean acid contents of the control and ammonium sulphate treatments are the same (5.71 and 5.79 per cent.), while in 0.44 of daylight organic acids accumulate in the manured leaves, i.e. a content of 6.19 as against 5.46 per cent. in the control.

# DISCUSSION

Since the pioneer work of Schulze and Prianishnikov many investigators have compared the changes in the nitrogen fractions which take place in the light in the dark. It has been repeatedly shown that plants growing with an

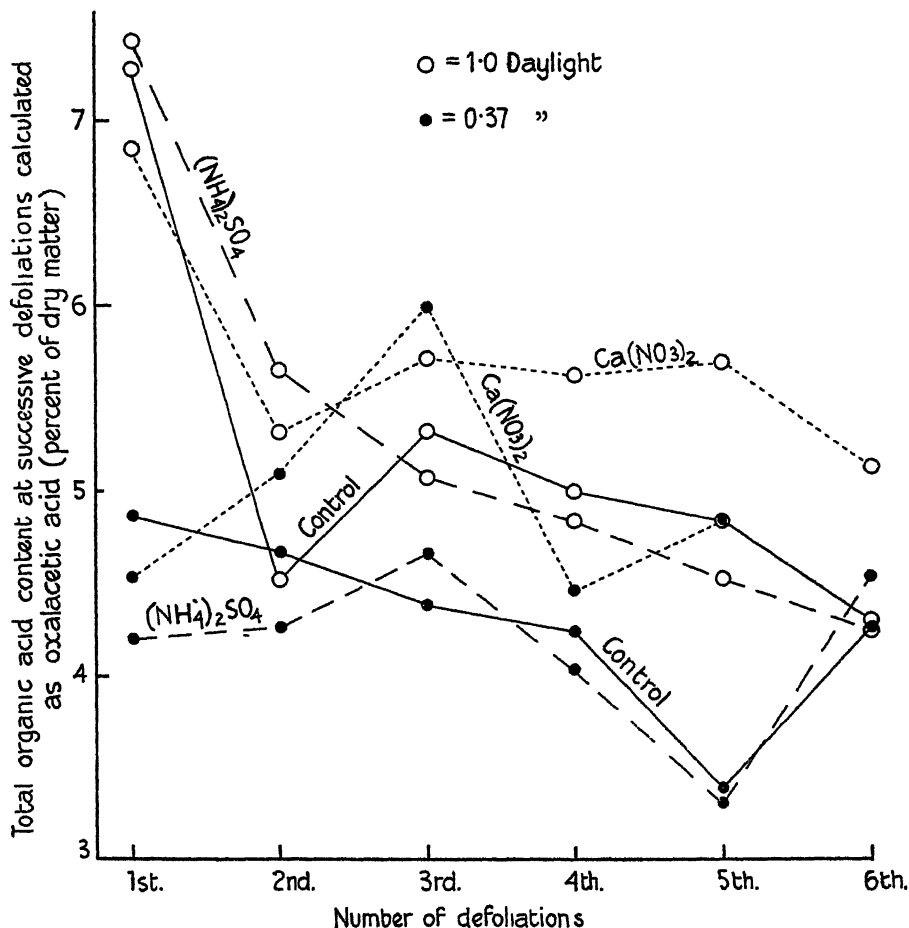


FIG. 20. Experiment II. The effects of light intensity and of nitrogen supply on the total organic acid content of the leaves of *A. tenuis* at successive defoliations.

adequate supply of light and nutrients will elaborate into protein the bulk of the nitrogen absorbed, while in the dark the protein level falls and there are accumulations of other nitrogen fractions. Yet in spite of these contrasting results little attention has been paid to the effects of varying light intensity on protein metabolism. It is true that a number of workers have investigated the effects of long and short days on the chemical composition of several plants. Their results, however, from the point of view of this investigation, are



complicated by the fact that whereas some of the treatments induced flowering others did not. It is therefore difficult to distinguish between the changes due to variations in day-length and the changes due to metabolic differences in the vegetative and the flowering phases. Nevertheless, the evidence of Pfeiffer (1926), Nightingale (1927), Tincker (1928), Zimmerman and Hitch-

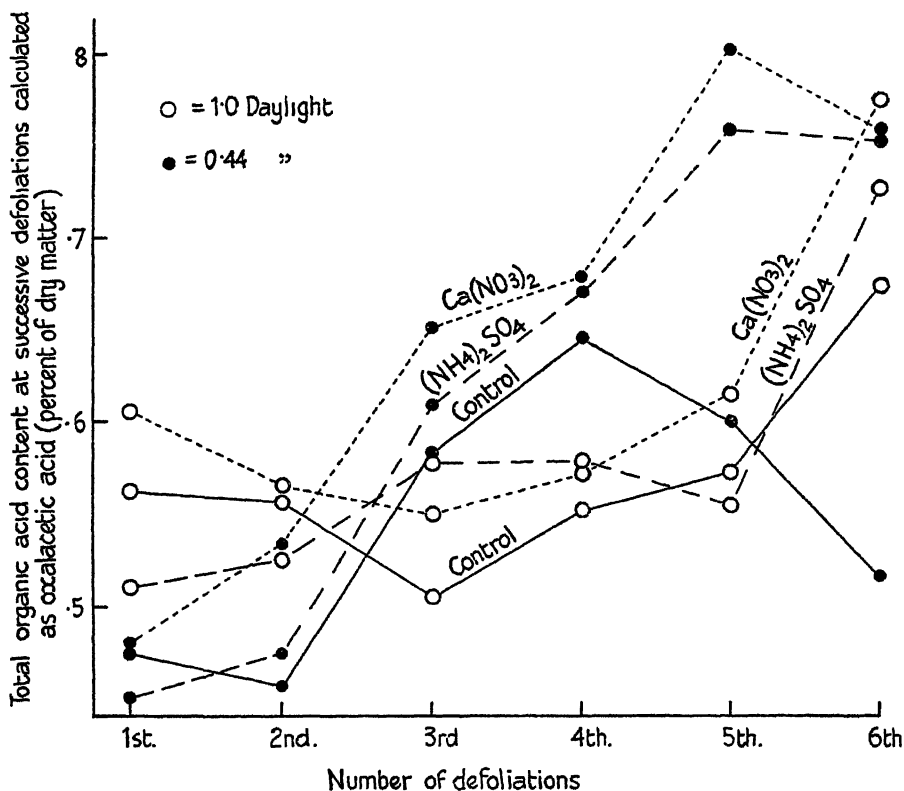


FIG. 21. Experiment III. The effects of light intensity and of nitrogen supply on the total organic acid content of the leaves of *F. rubra* at successive defoliations.

cock (1929), and Hopkins (1935) indicates that with short days plants contain more nitrogen and there is in particular, when they are fully manured, an accumulation of nitrates. The high nitrate concentration is not always correlated with the stoppage of growth following upon flowering. Hibbard and Grigsby (1934) observed that pea seedlings under short day conditions accumulated nitrates, while Nightingale *et al.* (1930) obtained similar results for the Biloxi soya bean when the day was so short as to inhibit flowering. Associated with this rise in nitrate nitrogen several workers have found that there is an increase in the carbohydrates, especially in starch.

Hopkins (1935) investigated not only the effects of short days but also the effects of shading on the soya bean with particular reference to the metabolism

of the nitrogen-fixing bacteria in the nodules. He observed that plants shaded (0.12–0.17 of daylight) for five to seven hours during the midday period decreased in carbohydrates and increased in all the nitrogen fractions. These changes, however, cannot solely be attributed to the direct effect of decreased light intensity on the plant, the possible indirect effects on the nodules must also be taken into account. The data of Arthur *et al.* (1930) also indicate that shading increases the total nitrogen content in a number of plants, while Kraybill (1923) found that with the peach and the apple decreasing the light intensity increased the total nitrogen and soluble nitrogen but depressed the carbohydrates.

The present investigation has shown that the effects of light intensity on the nitrogen content of the leaves are dependent upon the nitrogen supply. In the unmanured plants the results are in agreement with previous findings, for except in experiment I shading brought about increases in the nitrogen content. When the plants received ammonium sulphate reducing the light level first to 0.63–0.61 of daylight caused some increase in the total nitrogen, but a further reduction to 0.44–0.37 of daylight resulted in the shaded leaves containing the same amount of nitrogen as those grown in full daylight. On the other hand, in the calcium nitrate series the nitrogen content declined progressively as the light intensity was reduced.

The protein figures to a certain extent follow the same trends as the total nitrogen data. In full daylight the partition of the total nitrogen is normal, for most of the nitrogen is found in the form of protein, but there is some evidence that with high nitrogen supply the ratio of protein to total nitrogen falls. While in the four experiments the unmanured leaves contain on average 85.6–86.3 per cent. of their nitrogen as protein only, 63.9–85.1 per cent. of the accumulated nitrogen due to the addition of ammonium sulphate is found as protein and only 59.4–79.0 per cent. in the case of calcium nitrate.

In the unmanured series the increases in protein content of the shaded (0.44–0.37 of daylight) over the unshaded leaves follow the corresponding increases in total nitrogen. Thus the ratio of protein to total nitrogen remains relatively unchanged—namely, 82.4–85.1 per cent. On the other hand, the rises in total nitrogen content due to manuring are not paralleled by similar increases in protein content. In the case of ammonium sulphate only 64.2–11.5 per cent. of the extra nitrogen found in the leaves is elaborated into protein. Moreover, the addition of calcium nitrate though it may increase the total nitrogen content may at the same time *depress* the protein level (experiments III and IV).

Of the non-protein fractions the concentration of nitrates is most susceptible to changes in both manuring and light intensity. In full daylight the unmanured plants show the low nitrate nitrogen contents characteristic of nitrogen deficiency. On the other hand, manured plants, especially when calcium nitrate is applied, tend to accumulate nitrate nitrogen and the concentration reached is on occasion more than 0.9 per cent. of the dry matter.

Similar concentrations following on manuring have been reported for grasses by Eggleton (1935) and Blackman (1936); for tomato by Nightingale, Schermerhorn, and Robbins (1928), Clark (1936), and Wall (1939), and for pineapple by Sideris *et al.* (1938).

Irrespective of the nitrogen supply, shading brings about a marked increase in the nitrate content. In the unmanured plants where in full daylight the concentration is low, shading increases the concentration as much as five times, while in the manured series it is doubled. The other non-protein fractions show less marked differences between high and low light intensity. There are no substantial increases in amino or ammonia nitrogen in the shaded leaves, but there is evidence in experiment III that both shading and manuring may increase the amide content.

From the foregoing discussion it is clear that though at all light levels the nitrogen content of the leaves is increased by manuring yet the elaboration of this nitrogen is dependent upon the light intensity. Some insight into the possible factors responsible for this variation in the partition of the nitrogen can be obtained from the data concerning the differential effects of ammonium sulphate and calcium nitrate. In all four experiments while the leaves in full daylight gained an equal amount of nitrogen over the controls irrespective of whether ammonium sulphate or calcium nitrate was applied, yet at the lowest light intensity the gain was greater when ammonium sulphate was added. The extent to which such differences in nitrogen content are reflected in the partition of the accumulated nitrogen is seen in Table XII.

TABLE XII

*The Influence of Light Intensity on the Differential Partition of the Nitrogen Accumulated in the Leaves of Plants Manured with Ammonium Sulphate and Calcium Nitrate*

Experiments I-IV	Mean differences in content of nitrogen fractions due to manurial treatments (ammonium sulphate content less calcium nitrate content) (per cent. of dry matter)		
	Light intensity		Significant difference ( $P = 0.05$ )
	Daylight	0.44-0.37 Daylight	
Nitrogen fractions			
Total nitrogen . . . . .	-0.002	+0.283	0.148
Protein nitrogen . . . . .	+0.037	+0.289	0.098
Nitrate nitrogen . . . . .	-0.031	-0.084	0.035
Organic non-protein nitrogen . . . . .	-0.008	+0.078	0.081

The data in Table XII indicate that in full daylight the composition of the leaves is little affected by the source of nitrogen. In contrast at the lower light intensity the addition of ammonium sulphate results in a higher total nitrogen content. At the same time this increase is associated with an equal

rise in the protein content while the small gain in organic non-protein nitrogen is offset by the greater accumulation of nitrates in the plants receiving calcium nitrate. Since to the soils in which the plants were grown abundant calcium carbonate had been added the difference between the ammonium sulphate and calcium nitrate treatments can hardly be attributed to the calcium added in the latter. It seems more probable that such differences are associated with the absorption of the added nitrogen, for in the case of calcium nitrate nitrate ions only will be absorbed, while with the ammonium sulphate treatment both ammonium and nitrate ions will be taken up since the added ammonium nitrogen will in part have been converted to nitrates in the soil. It would appear from Table XI that in full daylight the elaboration of either ammonium or nitrate nitrogen into organic nitrogen takes place with equal facility. In 0.44-0.37 of daylight, however, while ammonium nitrogen is apparently synthesized into protein, nitrates tend to accumulate in the tissues unchanged. It might be suggested that if there had been no nitrification in the soil and a larger amount of ammonium ions had been absorbed the organic nitrogen level would have been higher and the divergence from the calcium nitrate data still greater.

Before, however, the part played by light intensity in the reduction of nitrates can be evaluated some consideration of the other factors responsible for nitrate accumulation will be necessary. Nightingale *et al.* (1930) and Wall (1939) found that tomato plants deficient in potassium stored nitrates, while Eckerson (1931*a*) observed similar high concentrations as a result of phosphorus starvation. Hibbard and Grigsby (1934) have demonstrated that pea seedlings growing in media, low in calcium or potassium, contain considerable amounts of nitrate nitrogen, while Richards and Templeman (1936) state that limiting the supply of phosphorus and more particularly potassium, leads to the accumulation of nitrates in the leaves of barley.

The chain of reactions responsible for the conversion of nitrate to ammonia is as yet not fully understood. Corbett (1935) has demonstrated that in the oxidation of ammonia by nitrifying bacteria both hyponitrous acid and hydroxylamine are produced. Chibnall (1939) has suggested that in plant cells nitrate reduction proceeds as follows: nitrate, nitrite, hyponitrous acid, hydroxylamine, ammonia. The presence of nitrites has been detected in numerous plants including grasses (Eggleton, 1935), and Eckerson has made a considerable study of the mechanism of nitrate reduction. She first showed (1924) that under carefully controlled conditions it was possible with sap expressed from plants to convert nitrate to nitrite *in vitro* and concluded that the rate of reduction could be taken as a measure of the activity of enzyme reducing system—reductase. On the basis of such tests Eckerson (1924, 1931, 1932) and Dittrich (1931) found that roots contained abundant reductase, but that it was also generally present but to a lesser extent in both stems and leaves. Other experiments of Eckerson indicated that shading, and deficiencies of nitrogen, phosphorus, potassium, and possibly the sulphate radicle, all

caused a reduction in activity. Nightingale *et al.* (1930, 1931) also claim that plants deficient in potassium and calcium lack reductase. Moreover, Eckerson (1931a) found that the reductase activity in the apple varies with the season and is greatest in the spring.

It would thus appear that a surprising large number of factors are responsible for limiting either the production or activity of reductase. That all these factors are directly concerned in the formation of the enzyme system seems improbable. Rather the results suggest that failure to detect nitrate reduction in the expressed sap does not preclude the possibility that the reduction will take place in the living cell. Again, the inability of plants to elaborate nitrate even in the presence of abundant carbohydrates cannot be ascribed solely to the absence of reductase, for as Richards and Templeman (1936) and Wall (1939) have pointed out, this could be brought about by mass action due to the accumulation of intermediate products. Moreover, it has been shown by Vickery *et al.* (1933, 1937), McKee and Lobb (1938), and Pearsall and Billimoria (1937, 1939) that leaves either originally free from nitrates or supplied with ammonium salts will produce nitrates under varying conditions. The nitrate concentrations, therefore, within the plant will be dependent upon the relative rates of (i) absorption of nitrates, (ii) reduction of nitrates, and (iii) oxidation of ammonia to nitrate. On this basis it would seem that since the unmanured plants in full daylight contained very little nitrate nitrogen, the rate of nitrate elaboration was in excess of that of nitrate absorption. The accumulation of nitrate in the manured plants, however, indicates that absorption took place at a faster rate than conversion. On the other hand, the markedly higher concentrations of nitrates in 0.44–0.37 of daylight, even in the unmanured plants, points to a decrease in the rate of nitrate conversion, since it is highly improbable that the nitrate absorption was increased by shading. In fact Weissmann (1925) found with cereals that the uptake per plant of phosphorus and potassium was greatly decreased by a reduction in light intensity, while Panchaud (1934) showed that the 'total mineral' uptake by *Raphanus sativus* was also depressed by shading. With tomatoes grown in a greenhouse Porter (1937) observed that at a quarter of the normal light the total salt (ash) uptake was 16 per cent. less than in the unshaded and larger plants. Similar decreases have been obtained by one of us in experiments still in progress on the uptake of nitrogen, phosphorus, and potassium by *Scilla nutans* grown in the field and in light intensities ranging from full to 0.2 daylight.

Although in all the investigations cited mineral uptake on a plant basis was checked by shading, the actual content of the tissues was not depressed but rather increased. It is highly unlikely, therefore, that in the present experiments the effect of shading on the accumulation of nitrates is due to the shaded leaves containing limiting amounts of those elements apparently essential for rapid nitrate reduction. That such elements, namely phosphorus, potassium, sulphur, and calcium were ever deficient is improbable on other grounds. In

the first place in full daylight leaf production was largely controlled by nitrogen supply and not by other mineral deficiencies (see Fig. 1). In the second place the soil used was a fertile one and in addition very appreciable quantities of calcium carbonate and superphosphate were added initially.

Although light intensity and nitrate conversion are apparently directly linked, in these experiments light does not always appear to be an essential factor. Nightingale and Robbins (1928) found that both *Narcissus Tazetta* and asparagus could convert nitrates into protein in the dark. That light even of low intensity (850–1150 m.c.) has an accelerating effect is put forward by Pearsall and Billimoria (1939), who worked with daffodil leaves floating on a solution containing ammonium nitrate and glucose. From their observations it would appear that the acceleration is greatest in the young green tissue and least in the white basal meristematic portion. That nitrate conversion is most active in the green portions of the pineapple leaf is also concluded by Sideris *et al.* in 1938. Pearsall and Billimoria (1939) also obtained evidence that the rate of ammonia nitrogen synthesis was greater in the light, while Dastur *et al.* (1938) claim that the increase in water-soluble nitrogen during exposure to light is dependent upon the quality of the light source. Their results must, however, be treated with considerable reserve on account of both their experimental procedure and the methods of chemical analysis. In the first place whole plants were exposed to the light but only the leaves taken for analysis (Dastur and Samant, 1933). Secondly the leaves, dried at 55° C., were extracted with cold water and all analyses carried out on this solution only. For the total nitrogen estimations no precautions were apparently taken to include nitrates and their technique, for the estimation of amides by conversion to ammonia would not distinguish between amide ammonia and any true ammonia nitrogen present. Moreover, since the plants prior to the experiment were kept in the dark for seventy-two hours the initial ammonia content may have been considerable. Nevertheless, in spite of these errors the marked variations in the gain in water-soluble nitrogen observed indicate that light quality may have had some effect on the synthesis of inorganic nitrogen, but as to the nature of this effect the evidence presented is insufficient for any conclusion to be drawn.

The investigations of Pearsall and Billimoria (1937, 1939) have, however, brought out another important aspect of nitrate reduction. They found that when leaves were floated on a solution of glucose and salts of inorganic nitrogen the amount of nitrogen absorbed was always greater than that found in the tissues. Since they observed that there was no discrepancy when organic sources of nitrogen were used in the external solution, they concluded that there was a loss of gaseous nitrogen due to the nitrite, formed during the reduction of nitrate, reacting with the amino acids. Mothes (1938) carrying out similar experiments could, however, detect no loss of nitrogen when the external solution contained nitrates, but found that if nitrite was substituted for nitrate then the loss was large. He concluded that in the nitrate series any

nitrite formed during the reduction of nitrates must be removed so quickly as to prevent reaction with the amino acids. The reaction cannot always be by any means instantaneous, for otherwise nitrites could not be detected and certainly could not accumulate (Eggleton, 1935) in the tissues of plants. In fact, Barritt (1931) showed that the nitrites formed by nitrifying bacteria did not react with amino acids added to the medium unless the pH of the culture solution was below 6.0. Increasing the acidity, however, led to a greatly accelerated loss of nitrogen. In this respect the leaves of daffodil employed by Pearsall and Billimoria had a sap of pH 5.2–5.4, while although Mothes did not determine the pH of the sap at least three of his plants, runner bean, tobacco, and maize, have saps whose pH is generally below 6.0 (Hurd-Karrer, 1939). Moreover, Mothes used solutions of a higher external pH than Pearsall and Billimoria and on the evidence of Wadleigh and Shive (1939) this difference should lead to greater content of organic acids and a slightly more acid sap. It seems unlikely, therefore, that the discrepancies between the two investigations can be ascribed to differences in hydrogen-ion concentration in so far as the pH of expressed sap is a measure of the acidity within the living tissues.

In Pearsall's experiments, even in the light and in spite of the external supply of glucose, the leaves failed to gain weight and moreover, particularly in the older half of the leaf protein, degradation occurred. From an examination of their data it would seem that the loss of nitrogen was more or less proportional to the amount of protein breakdown and this in turn was presumably associated with an increase in the amino acids. Mothes' experiments, however, were carried out in diffuse light and if this refers to diffuse daylight the light intensity would be higher than that employed by Pearsall. With the higher light level protein synthesis rather than protein breakdown might occur and thereby restrict gaseous nitrogen losses. Unfortunately this cannot be verified, as Mothes provides no data concerning the changes in protein nitrogen. Nevertheless, Mothes used for many of his experiments maize and wheat seedlings potentially capable of active protein synthesis, and akin to the basal portions of Pearsall's leaves which showed the minimum loss of nitrogen. In the present experiments active protein synthesis took place only in full daylight and in consequence there may have been little loss of nitrogen during the reduction of nitrate. On the other hand, in 0.44–0.37 of daylight, conditions unfavourable for protein elaboration, nitrogen losses might have occurred. Thus the difference in nitrogen content (Table XII) at the lower light level between the ammonium sulphate and calcium nitrate treatments could in part be ascribed to a greater loss of nitrogen from the plants receiving calcium nitrate, since in these the conversion of nitrates to protein is retarded, and the probability of loss greater. Alternatively it can be postulated that reducing the light intensity differentially affects the rates of absorption of ammonium and nitrate ions. On this point the evidence is conflicting. Pearsall and Billimoria (1939) found that relative to ammonium more nitrate

ions were absorbed in the light than in the dark. Marthaller (1937) states, however, that the uptake of ammonia compared to nitrate nitrogen by *Rumex obtusifolia* is greater in the light than in the dark.

These experiments have shown that shading brings about a considerable reduction in the water-soluble carbohydrate content of the leaves. In view of Paesch's (1935) contention that the protein level is largely controlled by the concentration of the chemically active forms of carbohydrate, and since moreover the conversion of nitrate to ammonia is an endothermic reaction, it might be put forward that the failure of the shaded leaves to elaborate protein is associated with their low carbohydrate concentration. Examination of the data, however, reveals no such relationship. For example, in experiments II-IV the shaded leaves of the unmanured plants, in spite of their reduced carbohydrate content, contain a higher percentage of protein and an equal absolute amount of protein to those of the plants grown in full daylight. Again, at the lower light levels the ability or inability of the manured plants to synthesize protein from the extra nitrogen absorbed is not significantly correlated with the carbohydrate concentration. The data of experiment IV illustrate the apparent independence of the two variables. In 0.63 of daylight plants receiving calcium nitrate contain as much or more protein than the controls over a range of from 3.06 to 5.45 per cent. of carbohydrates. Whereas in 0.44 of daylight comparable leaves with a carbohydrate content of 3.02-4.90 per cent. contain *less* protein than the controls. Similarly in experiments I-III the manured plants may contain more or less protein than the controls within a range of 1.9 to 5.4 per cent. of carbohydrates. Thus there is no evidence that there is a minimal concentration below which carbohydrates are not available for protein synthesis. Neither do the results support the conclusion of Paesch (1935) that there is a direct correlation between protein level and available carbohydrates irrespective of other factors. Rather they confirm the findings of Yemm (1935), Gregory and Sen (1937), and Richards (1938) that protein level is seldom directly related to sugar concentration.

While in shaded leaves manuring is not associated with a depression of the carbohydrates, additional nitrogen in full daylight leads not only to an increase in protein but also a reduction in carbohydrates. Before, however, considering this relationship the part played by organic acids must be taken into account. On the basis of the two schema for the protein cycle put forward by Gregory and Sen (1937), and Chibnall (1939), the sugars must be regarded as reserves for the formation of organic acids which alone are directly concerned in protein synthesis and decomposition. In attempting, therefore, to evaluate the relationship between proteins and carbohydrates the fluctuations in organic acids must also be considered. With this end in view the data of experiments II and III have been treated statistically by calculating within each light intensity the correlation between the gains or losses, following on manuring, in protein nitrogen, total water-soluble carbohydrates and total organic acids. The results are given in Table XIII. It is seen that in experiment II there is,



in the shaded leaves, a significant correlation between the increases in protein due to manuring, and changes in the carbohydrate content, whereas in daylight this relationship only holds when the organic acid effect has been eliminated. There is, however, a marked difference between the two light intensities, for while in daylight the protein increase is correlated with a carbohydrate *decrease*, in 0.37 of daylight the smaller protein gains are associated with carbohydrate *increases*. In contrast to experiment II the protein sugar correlations for experiment III are not significant.

TABLE XIII

*The Correlation between the Gains or Losses relative to the Control in the Protein Nitrogen (P), Total Water-soluble Carbohydrate (C), and Total Organic Acid (A) Content of Nitrogenously Manured Plants*

Experiment II.	Values of correlation coefficients.				
	PC	PA	AC	PC.A	PA.C
1.0 Daylight . . .	0.561	0.076	-0.270	<b>0.630</b>	0.284
0.37 „ . . .	<b>0.632</b>	-0.128	0.111	<b>0.655</b>	-0.258
Experiment III.					
1.0 Daylight . . .	0.061	0.573	0.006	0.082	0.574
0.44 „ . . .	-0.171	-0.420	0.083	-0.150	-0.440

Significant correlations ( $P = 0.05$ ) are given in heavy type.

The data in Table XIII also demonstrate that the protein changes relative to the control in the manured plants are not either in the shaded or unshaded leaves apparently correlated with the fluctuations in organic acids. The results for experiment IV also support this conclusion. At the three light levels within each manurial treatment the organic acid trends and concentrations are similar (Fig. 22), whereas the protein (Fig. 8) and carbohydrate levels (Fig. 18) are greatly affected by alterations in the light intensity. This lack of correlation does not, however, invalidate the conclusions of Gregory and Sen (1937) or Chibnall (1939) that the organic acids are a vital link in the protein cycle. Although the total organic acid may show little relationship with the protein changes it does not follow that the individual acids may not play an active part. It has been demonstrated, for example, by Pucher *et al.* (1937) that in respiring tobacco leaves, although the total organic acid content remains unchanged, citric acid increases and malic acid decreases. Unfortunately it is only in the tobacco leaf that there is, on occasion, a complete picture of the nitrogen, carbohydrate, and organic acids fractions (Vickery *et al.*, 1937). With other plants, e.g. tomato (Clarke, 1936), maize (Wadleigh and Shive, 1939), buckwheat (Pucher *et al.*, 1939) and barley (Yemm and Sommers, cited by Chibnall, 1939), it is not possible by present methods to identify and estimate a considerable proportion of the constituent acids so that the interpretation of the results is thereby hindered.

Apart, however, from the general role played by organic acids in protein

metabolism there is the question of the differential effect of manuring with calcium nitrate and ammonium sulphate. It has been demonstrated in experiments II-IV that within each light treatment the highest content of

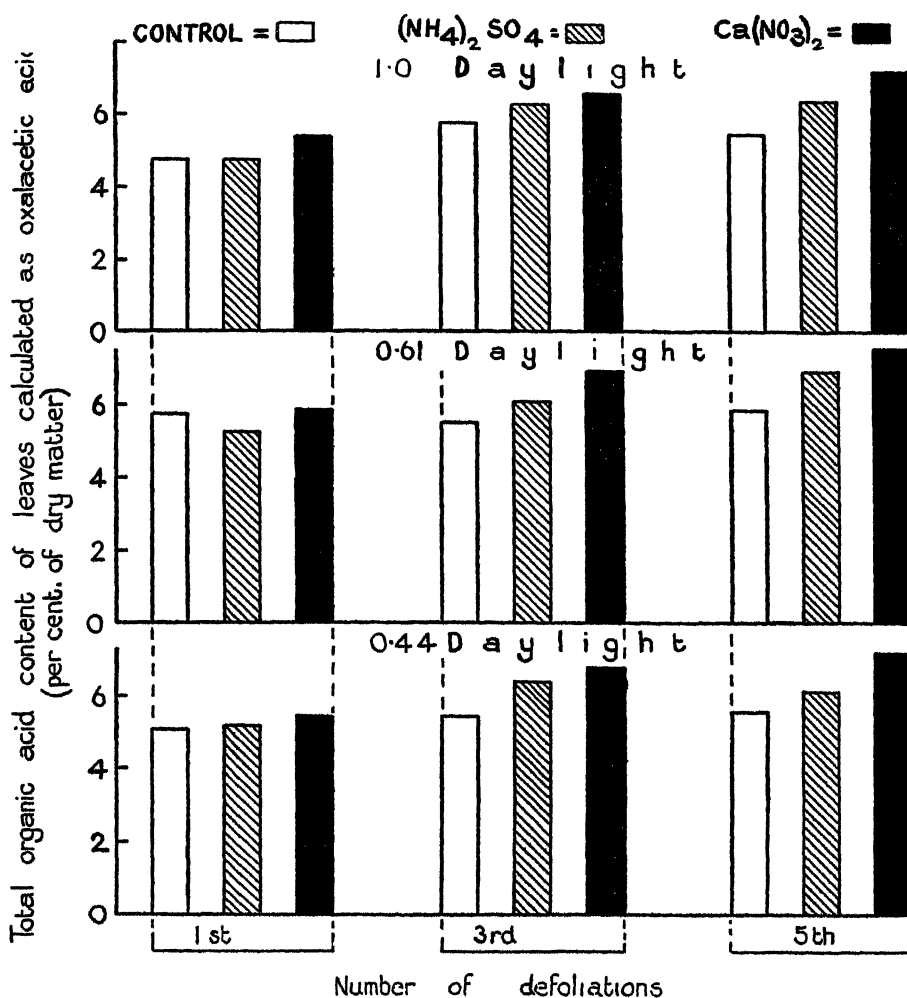


FIG. 22. Experiment IV. The effects of light intensity and of nitrogen supply on the total organic acid content of the leaves of *A. tenuis* at successive defoliations.

total organic acids occurs in the plants receiving calcium nitrate. The values for the ammonium sulphate treatment are intermediate and the controls contain the least amounts (see Figs. 19-22).

That tomato plants grown in cultures containing nitrate nitrogen have a higher total organic acid content than plants given ammonium nitrogen has also been observed by Clark (1936). Similarly, Wadleigh and Shive (1939) found that the total acid content of maize receiving only nitrates is higher

than that of plants receiving both ammonium and nitrate ions. They also showed that the total organic acid content increased as the external solution was made more alkaline. Finally Pucher *et al.* (1938) claim that the total organic acid content is related to the concentration of kations within the plant. They conclude that fluctuations in the content of kations is correlated with variations in the organic acids and that the hydrogen-ion concentration of the sap is controlled more by the organic than inorganic acids. On such a basis it is to be expected that plants in nitrate cultures will have higher organic acid contents than those receiving either ammonia and nitrate nitrogen or ammonium ions alone. In the nitrate series the uptake of nitrate anions will involve the uptake of kations such as calcium and potassium. In the ammonia series the ammonium absorption will be balanced by the absorption of anions such as sulphates and chlorides. As a result the conversion of the ammonia and nitrate nitrogen to protein will have opposite effects. The synthesis of nitrates will leave a surplus of kations and bring about an increase in alkalinity unless there is either an increase in the organic acids, a fresh uptake of anions, or an excretion of kations. The synthesis of ammonium ions will produce an excess of anions which will cause a rise in acidity unless the organic acid level falls, further kations are absorbed, or anions excreted. If, on the other hand, ammonium and nitrate ions are both absorbed and synthesized at the same rate the acidity will remain unchanged.

On such an hypothesis it is expected in the present experiments that the rise in organic acids following on manuring with calcium nitrate would be correlated with the increase in either the protein or total organic nitrogen, if the amount of elaborated nitrogen is some measure of the unbalanced kations left in the whole plant after the synthesis of the nitrate anions. It is seen, however, in the upper half of Table XIV that the correlation coefficients relating the increase in organic acids with either protein or total organic nitrogen are not significant. On the other hand, there is evidence particularly in experiment II that the total organic acid changes are linked with the accumulation of nitrates. Further confirmation of this relationship is given in the bottom half of Table XIV, where the data for both the ammonium sulphate and calcium nitrate treatments has been combined together.

The correlation between nitrate accumulation and the rise in organic acids is unexpected. It seems improbable that the nitrate content is a better measure of the free bases than the increase in elaborated nitrogen. Rather it would appear that this correlation is independent of any base accumulation, but what the relationship is remains obscure. It is clear, however, from the present experiments that the organic acids cannot solely be concerned with the ammonia protein cycle. The extremely variable effects of light intensity on the total organic acid content suggest that the nature and the proportions of the individual acids may be influenced by light intensity. Furthermore, it is also possible that the effects may be dependent on the nitrogen supply. At the lowest light intensity it has been seen that plants receiving calcium

nitrate may contain less protein than the controls. Since there is no evidence that carbohydrate or total organic acid levels were factors limiting protein synthesis, the apparent breakdown in the mechanism of nitrate reduction may be due to the absence of some particular acid or acids brought about by high nitrogen supply and low light intensity.

TABLE XIV

*The Correlation between the Gains or Losses, Relative to the Control, in the Total Organic Nitrogen (O), Protein Nitrogen (P), Nitrate Nitrogen (N), and Total Organic Acid (A) Contents of Leaves from Nitrogenously Manured Plants*

*Calcium nitrate data*

		Values of correlation coefficients					
Experiment II.	NA	OA	PA	PN	ON	NA.P	NA.O
1.0 Daylight	<b>0.905</b>	0.509	0.541	0.519	0.578	0.869	0.870
0.37 "	0.860	0.622	0.536	0.188	0.187	0.916	<b>0.967</b>
Experiment III.							
1.0 Daylight	0.763	0.388	0.451	<b>0.845</b>	0.744	0.720	0.770
0.44 "	0.508	0.250	0.345	<b>0.867</b>	0.622	0.446	0.464

*Combined calcium nitrate and ammonium sulphate data*

		Values of correlation coefficients					
Experiment II.	NA	OA	PA	PN	ON	NA.P	NA.O
1.0 Daylight	<b>0.667</b>	-0.230	0.076	0.427	0.375	<b>0.703</b>	<b>0.832</b>
0.37 "	<b>0.800</b>	-0.169	-0.128	-0.364	-0.412	<b>0.815</b>	<b>0.813</b>
Experiment III.							
1.0 Daylight	<b>0.774</b>	0.414	0.573	<b>0.745</b>	<b>0.660</b>	<b>0.647</b>	<b>0.731</b>
0.44 "	<b>0.577</b>	-0.309	-0.420	<b>-0.875</b>	<b>-0.705</b>	0.475	0.529

Significant correlations ( $P = 0.05$ ) are given in heavy type

While this discussion has so far been concerned with the effects of light intensity and nitrogen supply on the changes in nitrogen, carbohydrate, and organic acid fractions, there remains the question of how far these changes can be related to the variations in leaf production. It has been shown that in full daylight additional nitrogen leads to an increase both in leaf production and in protein level, whereas at lower light intensities such manuring may result in a depression of both growth and protein content. In order to determine how closely protein content and leaf production are linked correlation coefficients have been fitted to the data, and these are given in Table XV.

TABLE XV

*The Correlation between Changes Relative to the Control in Leaf Production and Protein Content of Plants receiving Additional Nitrogen*

		Value of correlation coefficient			
		Experiment			
Light treatment		I	II	III	IV
1.0 Daylight	.	0.408	0.588	0.979	0.823
0.63-0.61 Daylight	.	—	—	—	0.686
0.44-0.37 "	.	0.339	0.532	0.709	0.357

Level of significance ( $P = 0.05$ ) = 0.576

The figures in Table XV show that in full daylight the correlation coefficients between the increases in protein content and the increases in leaf production following on manuring are significant except in experiment I. On the other hand, at the lowest light intensity the correlation only reaches a significant level in experiment III. It cannot therefore be concluded that in the shaded plants the depression in growth due to manuring is always directly linked with a decrease in the protein content. It is not therefore surprising that the differences in growth due to manuring with ammonium sulphate and calcium nitrate cannot be interpreted in terms of the protein differences (see Table XVI). All that the correlation coefficients do is again to illustrate the differential effects of high and low light intensity; especially in experiments III and IV where the correlations are significantly different.

TABLE XVI

*The Correlation between Differences in Leaf Production and Differences in Protein Content of Plants receiving Ammonium Sulphate and Calcium Nitrate*

Light intensity	Value of correlation coefficient			
	Experiment			
	I	II	III	IV
1.0 Daylight . . .	+0.247	-0.183	+0.557	-0.877
0.63-0.61 Daylight . . .	—	—	—	-0.647
0.43-0.37 „ . . .	+0.252	+0.725	+0.109	+0.720

Level of significance ( $P = 0.05$ ) = 0.811

Since under low light intensity there is no consistent association in the manured plants between growth and the ability or inability to synthesize protein, an explanation must be sought elsewhere of the interaction between light intensity and the differential effects on growth of ammonium sulphate and calcium nitrate. Under field conditions many investigators have shown that for a wide range of plants the addition of either nitrates or of ammonium salts causes similar effects. Experiments on grassland have demonstrated that there is an equal response to ammonia and nitrate nitrogen even in the spring when the nitrification of the added ammonia nitrogen is slow (Blackman, 1936). On the other hand, the results of previous experiments (Blackman, 1934) carried out in the early summer period are in agreement with the results obtained in the pot experiments that ammonium sulphate in daylight may result in a higher production of leaves from frequently defoliated plants (experiments III and IV). In contrast, several investigators have recorded that in pot or water cultures the growth of plants receiving nitrate nitrogen is markedly better. Beaumont *et al.* (1933) found that under low light intensity several species of grasses given ammonia nitrogen were stunted compared to plants supplied with nitrates. Harrison (1934) observed that the growth of *Poa pratensis* in cultures high in nitrates was superior to that in cultures high in ammonia nitrogen. Clark (1936) showed that tomato plants grown in a shaded greenhouse and receiving nitrate nitrogen were four times

as large as those given ammonia nitrogen. Dawson (1938) compared the growth of tobacco plants in ammonia and nitrate nitrogen and concluded that nitrate nitrogen was superior. These investigations, however, differed in the culture technique employed. Beaumont *et al.* adopted a method (Beaumont, 1929) in which no particular attention was paid to the aeration of the culture solutions, while Clark, Dawson, and Harrison used sand cultures and constant renewal of the solutions by the drip method. The importance of aeration as a factor in determining the relative merits of ammonia and nitrate nitrogen has been stressed by Arnon (1937). He observed that if the water cultures were infrequently aerated then barley seedlings invariably grew faster in the nitrate series. When, however, the solutions were well aerated this aeration had a larger beneficial effect on the plants receiving ammonium salts. Nevertheless, in spite of good aeration, it was only during the summer that the two sources of nitrogen gave equal growth; in the spring and autumn the nitrate manuring still produced larger plants. If, however, traces of copper and manganese were included in the culture solutions then there was little to choose between the two sources of nitrogen over the whole season, for these additions, more particularly in the spring and autumn, benefited to a large extent the plants in the ammonia nitrogen series. Finally, the researches of Marthaller (1937) have revealed that such differential nitrogen effects are dependent on the species. He found in water-culture experiments (without frequent aeration and no systematic control of pH) that with *Urtica dioica* and *Rumex obtusifolia* growth was equal in the two culture series when the nitrogen concentration was low, but with rising concentration the plants in the nitrate series grew faster. However, in the case of *Plantago media* as the concentration increased the size of the plants rose progressively in the ammonia series but fell in the nitrate solutions. Lastly, the relative growth of *Chenopodium album* in the two culture solutions was independent of the concentration; at all contents it was superior in the ammonium salt medium.

It is obvious from these investigations that there is no single factor governing the relative effectiveness of ammonium and nitrate salts as a source of nitrogen. Beaumont *et al.* (1933) concluded that the poor growth made in the ammonia series was due to a toxic accumulation of ammonia nitrogen in the plants, since they found in both the shoots and roots a considerable content of ammonia nitrogen. In contrast, Clarke (1936) demonstrated that irrespective of whether the tomatoes were given ammonia nitrogen at the full concentration or at one-third the concentration the growth was uniformly poor compared to that in the nitrate series. Moreover, the growth showed no relationship to the markedly lower ammonia nitrogen content of the plants grown in the more dilute solution. Arnon (1937) puts forward the suggestion that in poorly aerated solutions the oxygen in the nitrate radicle can be utilized by the roots and thus lead to better growth, for plants supplied only with ammonia nitrogen will be deprived of a supplementary source of oxygen. He ascribes the beneficial effects of copper and manganese in the

ammonium salt cultures to their catalytic activity in the oxidation-reduction processes within the plant and concludes that these activities offset the oxidative function of nitrates. On the other hand, Burström (1939) claims that manganese catalyses the reduction of nitrates.

In the light of this and other evidence it seems no longer possible to accept Prianishnikov's hypothesis that the injurious effects of ammonium salts are due solely to a toxic accumulation of ammonia nitrogen within the plants as a result of carbohydrate deficiency.<sup>1</sup> When leaves are kept in the dark, accumulation of ammonia nitrogen may take place before the complete exhaustion of the carbohydrates and while the tissues still contain appreciable amounts of organic acids. Yemm (1937) found that in barley leaves ammonia nitrogen began to accumulate after forty-eight hours' darkness and at this point, according to previous experiments, one-third to half the available carbohydrates would still not be catabolised (Yemm, 1935). Moreover, starch, sucrose, and glucose would still be present in measurable amounts when the ammonia nitrogen content reached a maximum. Furthermore, according to the findings of Yemm and Somers (quoted by Chibnall, 1939), the organic acid contents had not markedly fallen from their initial values. On the other hand, Vickery *et al.* (1937) observed that in the case of respiring tobacco leaves ammonia accumulation was not initiated until the carbohydrates were exhausted but occurred at a time when the total content of organic acids still amounted to 1.5 per cent. of the fresh weight.

That ammonia nitrogen may accumulate in the illuminated leaves of grasses is evident from the data of Petrie and Wood (1938, 1939). Their plants during the short experimental period were grown under constant conditions of temperature, humidity, and light (800 m.c.) but received varying amounts of ammonium salts and water. With increasing nitrogen supply the ammonia nitrogen content of the leaves of both *Phalaris tuberosa* and *Lolium multiflorum* rose sharply without any apparent deleterious effects. In the case of *P. tuberosa*, concentrations as high as 0.4 per cent. of the dry matter (9.3 per cent. of the total nitrogen) were observed in leaves containing 4.7 per cent. of sugars. In the leaves of *L. multiflorum* with a low water content the ammonia nitrogen amounted to 0.93 per cent. (22 per cent. of the total nitrogen), while the sugar content was 7.7 per cent. In fact, such concentrations have seldom been exceeded in plants kept in the dark and receiving ammonia nitrogen. The highest figure recorded by Burkhart (1938) subsequent to the exhaustion of the carbohydrates amounted to 0.087 of the fresh weight of buckwheat plants. Moreover, the ratio of ammonia to total nitrogen observed by Petrie and Wood is of the same order as in etiolated seedlings, mainly of legumes, which Prianishnikov and Schulow (1910) and Prianishnikov (1922) considered were suffering from 'ammonia poisoning'.

<sup>1</sup> For a full account of the work of Prianishnikov and his school reference should be made to the reviews by McKee (1937) and Nightingale (1937) and the recent book on protein metabolism by Chibnall (1939).

In this investigation the depression in growth caused by calcium nitrate under low light intensity indicates that other factors than ammonia nitrogen accumulation which are associated with increased nitrogen supply may be injurious. Even the depression brought about by ammonium sulphate is not correlated with an accumulation of ammonia nitrogen in the leaves (Table IX). It could, however, be put forward that a low ammonia nitrogen content in the leaves did not preclude the possibility of accumulation in the roots. Such a differential accumulation is, however, unlikely, for Beaumont *et al.* (1933), Clarke (1936), and Sideris *et al.* (1938) all found no marked unequal partition of ammonia nitrogen between root and shoot. Finally, the experiments of Marthaller (1937) provide similar evidence to the present results that the relative effectiveness of ammonia and nitrate nitrogen cannot be explained on the grounds of ammonia poisoning. In fact, on the basis of the evidence cited it is difficult to maintain that even in respiring leaves or etiolated seedlings ammonia nitrogen accumulation is the causal agent of injury or death. It is rather concluded that some factor or set of factors correlated with this accumulation brings about metabolic disorganization. For example, once the carbohydrate supply is exhausted there will be no substrate left for the formation of organic acids and a further accumulation of ammonia nitrogen will lead to rapid decrease in the cell acidity which may be deleterious.

Since under low light intensity there seems little ground for associating the growth of the manured plants with either the protein content or the accumulation of ammonia nitrogen the depression in growth might be ascribed to the accumulation of nitrates. The data, however, lend little support to the supposition that there is a limiting nitrate concentration above which a further rise causes a depression in growth. For example, in experiment II, when the nitrate concentrations ranged from 0.21 to 0.39 per cent. of the dry matter, additional nitrogen at the lowest light level brought about a progressive decline in leaf production. But in experiment I, where at the same light level nitrogenous manuring increased growth, the nitrate content varied from 0.17 to 0.54 per cent. Again, in experiment IV in full daylight and at the intermediate light intensity ammonium sulphate increased growth yet the nitrate concentrations in the leaves were 0.20-0.40 and 0.39-0.52 per cent. respectively. Concentrations in fact much in excess of those in experiment II where the growth of the shaded plants was depressed by the addition of ammonium sulphate. It could, however, be put forward that the critical nitrate concentration varied from experiment to experiment and thus that the greater diminution in growth caused by calcium nitrate was due to the higher nitrate concentration. Correlation coefficients between the relative growth and the relative nitrate concentrations of shaded plants receiving ammonium sulphate or calcium nitrate do not, however, reveal any significant relationship. For experiments I-IV the correlations are respectively -0.207, -0.051, -0.615, and 0.353, not one of which is significant.



Although the changes in the nitrogen fractions do not appear in all experiments to be directly linked with the differences in growth, they may yet act through their effects on the processes of assimilation and respiration. It has been shown that over the whole period shading reduces leaf production and that associated with this reduction there is in general a fall in the content of total water-soluble carbohydrates. This relationship is somewhat anomalous, for between the initial and subsequent cuts, reducing the light intensity may have opposite effects. Comment has already been made that in experiments III and IV (Figs. 17 and 18) shading initially leads to a gain in carbohydrates while subsequently it causes a loss. Precisely the same effect has been observed in the growth data, for while within each manurial treatment shading at first increased leaf production, yet in the following cuts production fell with diminishing light intensity (Blackman and Templeman, 1938). This interdependence is clearly shown in Fig. 23, where the growth and carbohydrate data of experiment IV are given for the first two cuts.

It is concluded that this reversal of the effects of shading is linked with the presence of reserve carbohydrates in the roots and stem bases. Since prior to the experimental period all the plants had been grown in full daylight, it is reasonable to suppose that some carbohydrates would have accumulated in the basal portions. That cutting may bring about a mobilization of these reserves has been demonstrated by Buckey and Weaver (1939), who found that the drastic defoliation of *Andropogon* spp. brought about a withdrawal of carbohydrates from the roots. In the present experiments it is postulated that this withdrawal is linked with the light intensity. At the time of the initial cut shading leads to the transference of carbohydrates from the roots to the shoots but in the unshaded plants the sugars are in part translocated away from the leaves. As soon, however, as under low light intensity these reserves of carbohydrates are exhausted both the production and carbohydrate content of the leaves will no longer show an anomalous relation to light intensity. From Fig. 22 it would appear that the reserves in the shaded plants are exhausted after the initial cut.

The results in Fig. 22 also indicate that subsequent to the initial cut leaf production and carbohydrate level are largely regulated by light intensity. Although the magnitude of the carbohydrate carbon depression is modified or even offset to a considerable extent by the smaller fluctuations in the total organic acids, yet the net decrease in the content of readily available carbon coupled with the decreased growth indicates that the assimilation rate is closely correlated with the light intensity. That the light effect should be so marked is somewhat surprising, for the majority of previous investigators have shown that halving the light intensity has little effect on growth or the assimilation rate. It must, however, be borne in mind that the present plants have been cut at short intervals while those of other workers have been allowed to grow unchecked. After each defoliation, therefore, new leaves must be produced under the different light intensities, and in this connexion the

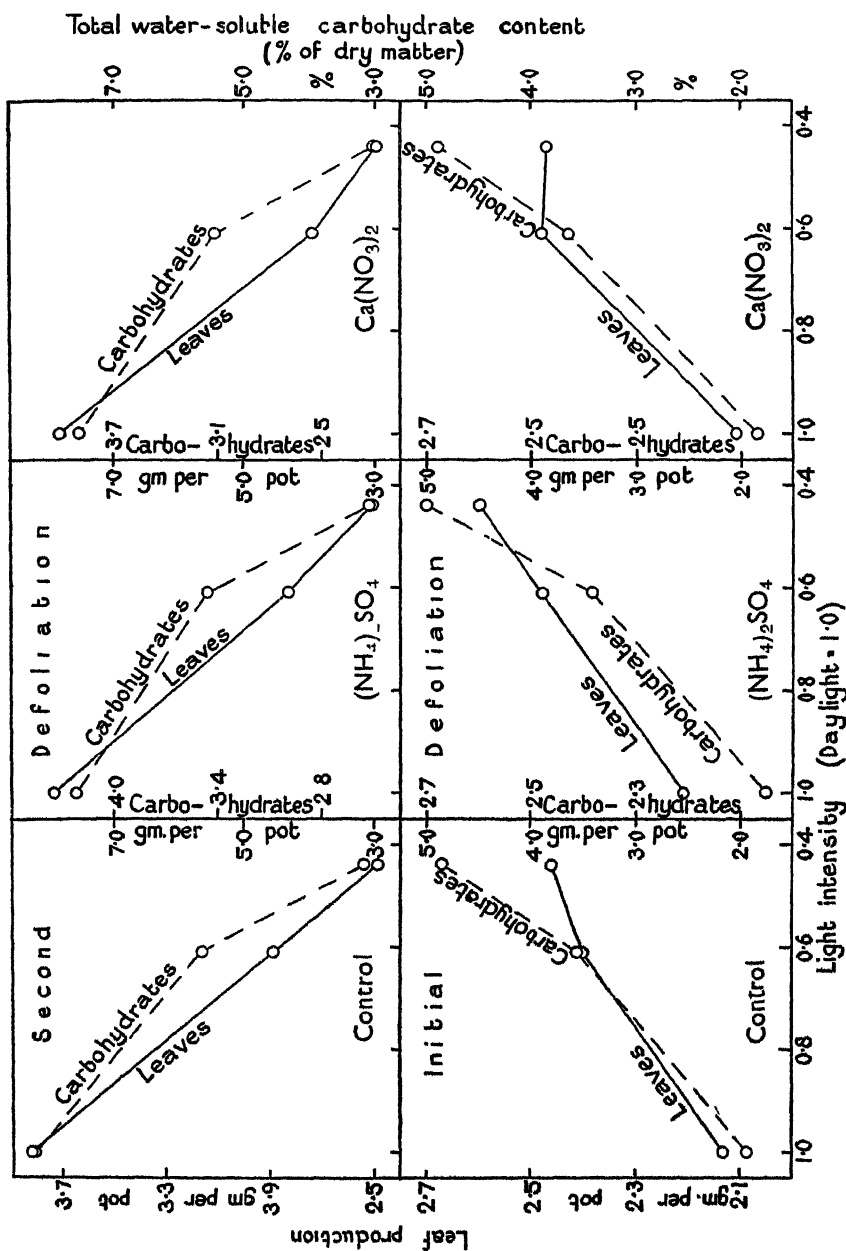


FIG. 23. Experiment IV. The influence of light intensity on the production and total water-soluble carbohydrate content of the leaves of *A. tenuis* at the time of the initial and second defoliation.

experiments of Gassner and Goeze (1934) are of interest. Using the young leaves of wheat and rye seedlings they showed that the maximum assimilation rate of leaves exposed to artificial light for twelve hours was reached in a much shorter time than in those given, for example, only two hours' light per day. Thus, in the present experiments the depression in leaf production brought about by shading may be due both to a decrease in the final assimilation rate and also to differences in the time taken under the various light intensities for this maximum rate to be attained.

In addition, Gassner and Goeze claim that whereas in high light nitrogen-starved leaves have a lower assimilation rate, yet there is no nitrogen effect in leaves illuminated for only three hours per day. That nitrogen deficiency depresses the assimilation rate of *Sinapis alba* is put forward by Muller and Larsen (1935). On the other hand, Crowther (1934) and Gregory (1937) could find no relationship between nitrogen supply and photosynthesis in the case of cotton and barley respectively. Crowther's and Gregory's plants were, however, grown in full daylight while both Muller and Larsen and Gassner and Goeze employed artificial light of lower intensities. Moreover, Gregory (1937) criticizes these workers on the grounds that at least in daylight nitrogen deficiency accelerates senescence, and this in turn is linked with a falling off in the assimilation rate. To what extent the rate of senescence is influenced by light intensity is not known, but it is clear that unless strictly comparable material is used for comparison false conclusions may be reached as to the effect of nitrogen deficiency on the assimilation rate.

Although the balance of evidence indicates that the net assimilation rate is unaffected by nitrogen deficiency it does not follow that the very high rates of nitrogenous manuring employed in this investigation may not have depressed assimilation, particularly in the shaded plants. It has, for example, been shown by Porter (1937) that shading reduces both the chlorophyll content and the growth rate of tomatoes, while Michael (1935) demonstrated that in the leaves of *Tropaeolum* changes in the chlorophyll were linked with changes in the protein level. Moreover, Gassner and Goeze (1934) found that assimilation rate, protein content, and chlorophyll concentration were intimately connected. Thus in experiments III and IV the depression in the protein content caused by additional nitrogen at the lowest light intensity may have brought about a fall in the chlorophyll level and thereby a decrease in assimilation rate which in turn lowered the growth rate. On the other hand, the results of experiment II cannot be explained on this basis, for here the depression in growth was associated with a gain in protein.

Under field conditions the rate of growth will be dependent on the excess of assimilatory products made during the day over the respiratory losses by the *whole* plant during the night. Therefore with varying light intensities the changes in growth caused by additional nitrogen will not only be linked with effects on assimilation but also with the effects on respiration. According to Gregory and Sen (1937) and Richards (1938) except in cases of extreme

potassium deficiency the respiration of barley leaves is largely correlated with their protein content. In this investigation shading has, in the unmanured plants, very considerably increased the protein level of the leaves and may therefore have also increased the respiration rate. Moreover, additional nitrogen by raising the protein content in experiments I and II may have caused a further rise in respiration, whereas in experiments III and IV the fall in protein due to manuring may have been associated with a diminution in respiration. It also follows that even though in full daylight the net assimilation rate is not affected by nitrogen supply (Crowther, 1934), yet if additional nitrogen leads to a higher content of protein and therefore a higher respiration rate then this rise must be offset by an increase in the assimilation rate.

From the foregoing discussion it is clear that a number of factors may operate in bringing about the changes observed. The great variation of the data from different experiments indicates that the relationships are exceedingly complex, but this complexity may, in part, be due to a lack of knowledge concerning the changes in the roots and their effects on the shoot. At the outset it was realized that data concerned only with leaf production were unsatisfactory. On the other hand, practical difficulties ruled out a detailed study of the roots. If at every cut pots had been withdrawn for root-weight determinations, the largest experiment would have involved the use of fourteen hundred pots and the smallest trial over seven hundred. Again it would have entailed the washing away of at least two tons of soil at each sampling and in a short space of time; neither the workers nor the facilities were available for this. However, at the conclusion of experiments III and IV it was possible to determine the root weight in a randomly selected quarter of each pot in half the replicates; the data thus collected is seen in Table XVII.

TABLE XVII

*The Effects of Light Intensity and Nitrogen Supply on the Growth of Leaves and Roots at the Time of the Final Cut*

Treatments.		Dry matter production (gm. per pot)					
		Experiment III.			Experiment IV.		
		Leaves.	Roots.	Leaves. Roots.	Leaves.	Roots.	Leaves. Roots.
1.0 Daylight	Control	1.75	8.87	0.20	2.00	5.51	0.35
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.81	7.90	0.73	5.61	3.58	1.56
	Ca(NO <sub>3</sub> ) <sub>2</sub>	4.55	6.07	0.75	4.44	2.67	1.66
0.61 Daylight	Control	2.03	3.96	0.51	2.40	2.05	1.17
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.93	2.64	1.11	3.10	1.58	1.96
	Ca(NO <sub>3</sub> ) <sub>2</sub>	1.96	3.45	0.58	1.98	1.46	1.36
0.44 Daylight	Control	1.13	3.93	0.29	1.66	1.43	1.16
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.94	2.29	0.41	1.07	0.76	1.41
	Ca(NO <sub>3</sub> ) <sub>2</sub>	0.48	1.13	0.43	0.62	0.61	1.01

From Table XVII it is clear that the general trend on a whole plant basis is similar to that obtained from the leaf results only. However, by the inclusion

of the root data the size of the increases in growth brought about by additional nitrogen in full daylight is reduced, while in contrast the depressions at the lowest light intensities are accentuated. These modifications are due to the effects of light intensity and nitrogen supply on the relative production of leaves and roots. In daylight there is a striking alteration in the leaf-root ratio following on nitrogenous manuring. It is at this light intensity that the ratio is highest for calcium nitrate, but in the case of the control and ammonium sulphate treatments the maximum value is reached at the intermediate light level.

It is considered that these results confirm the conclusion already reached that at low light intensities leaves are produced at the expense of the roots. That frequent defoliation combined with high nitrogen supply leads particularly to a great reduction in the roots and rhizomes of *Poa pratensis* has been established by Harrison (1934). In some of the experiments he found that close and repeated cutting brought about the death of the whole plant, especially where the nitrogen supply was kept at a high level. In contrast to the present results he observed that plants growing in solutions containing more ammonium than nitrate ions produced less roots and rhizomes than those given solutions with a preponderance of nitrate ions. Harrison concluded that the death of the rhizomes was due to the exhaustion of the carbohydrate reserves, which were preferentially utilized for leaf production. Thus the rate of exhaustion was accelerated by high nitrogen supply, since this initially increased the rate of leaf formation and thereby brought about a more rapid depletion of the reserves.

In the present experiments the preferential use of available carbohydrates for leaf production might account for the change in the leaf-root ratio caused by additional nitrogen in full daylight. But at the lowest light intensity the still greater depression in root production brought about by nitrogenous manuring cannot be explained on this basis, for in experiments III and IV additional nitrogen did not at any time increase leaf production. Nor from the analytical data can it be concluded that manuring caused a greater depletion of available carbon, for if the carbohydrate and organic acid levels are both taken into consideration then in experiments III and IV the manured leaves in 0.44 daylight contained rather more available carbon than the unmanured. Such data do not, however, rule out the possibility that carbohydrate depletion in the roots did occur, for high nitrogen supply might, for example, have led to a rise in the root respiration rate.

In conclusion it seems evident that the effects of light intensity and nitrogen supply on leaf production are in part dependent upon their effects on root development. It is not therefore surprising that the changes in leaf production cannot wholly be interpreted on analytical data obtained only for the leaves. In any future research for a fuller interpretation a knowledge not only of the root changes will be necessary but also information concerning the respiration and assimilation rates. Moreover, a study of the roots may throw some light

on the problem of the relationship between light intensity and nitrogen metabolism in the leaves, for in this respect the shoot and the root changes may be linked. Finally the frequency of defoliation may also be an important factor. It is by no means certain, for example, that undefoliated plants would give the anomalous results obtained in these experiments.

#### SUMMARY

In a previous investigation it was demonstrated that while in full daylight the addition of ammonium sulphate or calcium nitrate markedly increases the leaf production of frequently defoliated grasses (*Agrostis tenuis* and *Festuca rubra*), yet at lower light intensities (0.63–0.37 of daylight) the same manurial treatments may depress leaf production. Moreover, the depression is greater in the case of calcium nitrate. In the present investigation an attempt has been made to correlate the growth changes with the effects of light intensity and of nitrogen supply on leaf metabolism. For this purpose the leaves have been analysed for total nitrogen, for protein, and for amino, amide, residual, ammonia, and nitrate nitrogen, total water-soluble carbohydrates and total organic acids—in all 1,200 separate determinations.

In full daylight it has been found that whether ammonium sulphate or calcium nitrate is added there is an equal gain in total nitrogen largely in the form of protein. At the lower light intensities, although there is a rise in total nitrogen due to manuring, elaboration of protein is reduced as the light intensity falls and nitrates in particular accumulate. In fact, at the lowest light intensity (0.44–0.37 of daylight) the protein level may fall below that of the unmanured plants even though the controls contain less total nitrogen. Under shade conditions, but *not in full daylight*, plants receiving ammonium sulphate contain more total nitrogen, more protein and less nitrates in their leaves than those given calcium nitrate. It would appear that with a high nitrogen supply shaded leaves lose their ability to elaborate nitrate rather than ammonium ions into organic nitrogen.

Shading reduces the carbohydrate content by 20–60 per cent., but the level of total organic acids is much less affected. In consequence the ratio of organic acids to carbohydrates is greatly altered by light intensity. In daylight the gains in protein following on manuring may be significantly correlated with a decrease in the carbohydrate content, but at the lowest light intensity manuring has little effect on the carbohydrate level. Irrespective of the light intensity, changes due to manuring either in protein or total organic nitrogen show no correlation with the total organic acid fluctuations. Under shade conditions the ability or inability of the leaves from manured plants to synthesize inorganic nitrogen is not dependent upon any critical concentration either of carbohydrates or of total organic acids. At all light intensities the addition of ammonium sulphate, and more particularly of calcium nitrate in general, leads to increases in the total organic acids; these increases are significantly correlated with the accumulation of nitrates.

In full daylight the increases in leaf production due to additional nitrogen are significantly correlated in three out of four experiments with the ability of the leaves to elaborate protein. At the lowest light intensity the depression in growth caused by manuring is generally not significantly correlated with the decrease in protein content. Neither is the depression in growth a function of ammonia or nitrate nitrogen accumulations, low carbohydrate content or total organic acid concentration. It is concluded that the effects of light intensity and nitrogen supply on leaf production and leaf metabolism may in part be controlled by their effects on root production and root metabolism. There is evidence firstly that these factors markedly affect the leaf-root ratio and secondly that at low light intensities leaf production takes place at the expense of the roots, since there is a transference of carbohydrates from the roots to the leaves when the plants are initially shaded. Finally, in the shaded plants it is possible that additional nitrogen may affect both the assimilation and respiration rates.

The authors are indebted to Imperial Chemical Industries for permission to publish some of the results of this investigation. They also wish to thank Mr. V. Martin for his help with the determinations of the carbohydrates and Mr. G. Stroud for his assistance in the estimation of the organic acids.

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# Heteroauxin and the Growth of Meristems of Brassica

BY

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With five Figures in the Text

## 1. INTRODUCTION

THE suggestion made by Howard (1938) that heteroauxin acts as a root-determiner was a very obvious one. A similar conclusion had in fact been reached independently by Fischnich (1938) from experiments with cuttings of *Populus nigra* var. *pyramidalis* and by Stoughton and Plant (1938) from experiments with root-cuttings of *Crambe maritima*.

Although all three sets of experiments are rather crude in that high concentrations of auxin were used, there is very good reason for believing that heteroauxin does act as a root-determiner. The question considered in this paper is how large a meristem has to be before it is definitely a shoot initial and not capable of being converted into a root by the action of heteroauxin.

## 2. PREVIOUS WORK

There seems to be no doubt that, as is stated by Priestley and Swingle (1929, p. 84), 'as a rule, when once the meristem cells are organised into a characteristic "Anlage" the organisation does not change its character whatever the food supplied to it'.

One exception to this rule is the behaviour of occasional buds on the root of *Rumex acetosella* under experimental conditions. The storage root normally grows horizontally. Beijerinck (1887) found that if the storage root was placed vertically, some of the lower buds would grow into roots instead of shoots. Edmondson (see Priestley and Swingle, 1929, p. 26) has confirmed these observations. This case is easily explained on the hormone hypothesis since in the storage root placed vertically there might be transport of the hormone to the lower end.

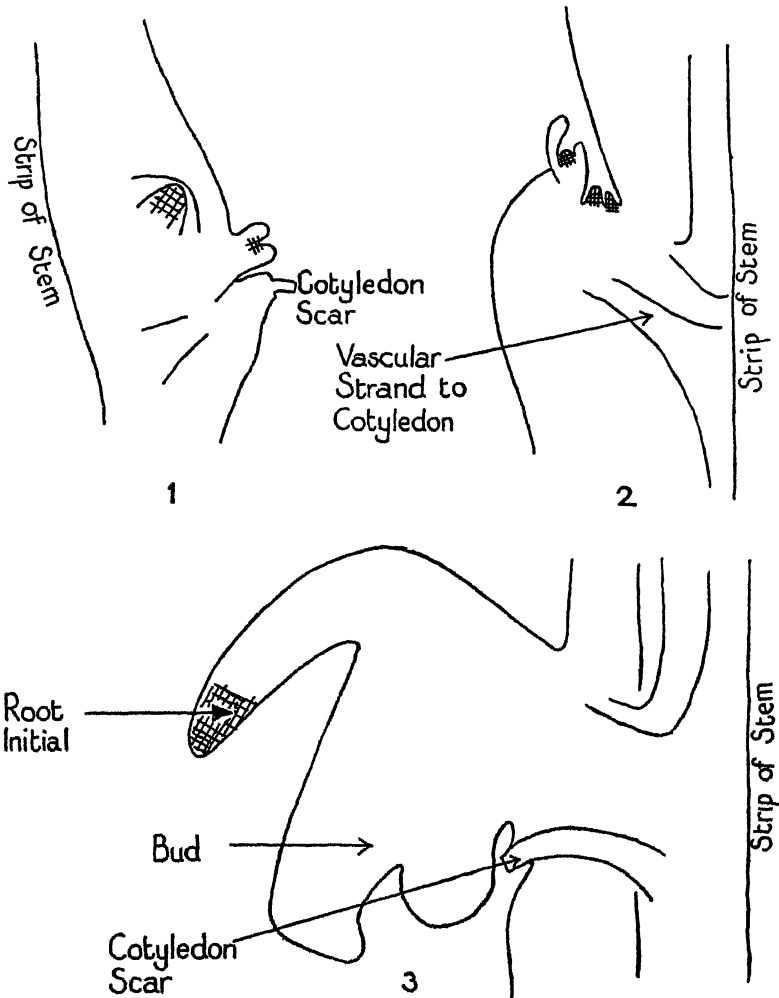
Carlson (1938) has described the growth of shoots from just behind the tip of the roots of *Pogonia ophioglossoides*. This appears to be the nearest approach to a root-tip meristem being converted into a shoot meristem.

## 3. GROWTH OF AXILLARY MERISTEMS IN BRASSICA

In a previous paper (Howard, 1938) it was suggested that it was possible for a whole leaf-initial to be converted into a root (see Fig. 12 of that paper).

Further consideration suggested that this was unlikely. The observation, if correct, would be an extremely important one and perhaps the most conclusive in showing the root-determining power of auxin.

An experiment was, therefore, carried out with seedlings of kale, *Brassica*

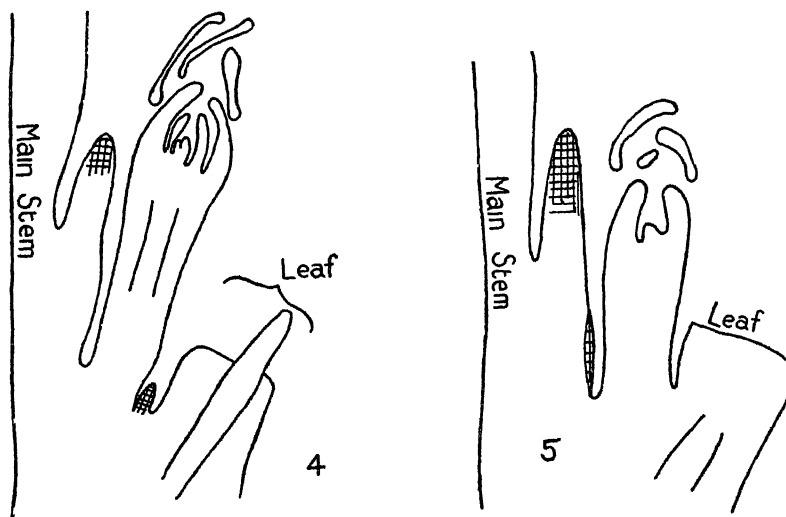


FIGS. 1-3. Kale (*Brassica oleracea*) seedlings. Longitudinal sections through axils of cotyledons. Meristems are shown hatched.

*oleracea*, to try and confirm the previous observation. Seedlings with two large leaves were decapitated and all axillary buds, except those in the axils of the cotyledons, were removed. The plants received a variety of auxin treatments, and samples of the axils of the cotyledons were fixed one, two, and three weeks after decapitation. These samples were embedded in wax

and sectioned longitudinally. In all the auxin treatments a smear of a 1 per cent. paste of indole-3-acetic acid in lanoline was placed on the stem of the seedling at a higher level than the axils of the cotyledons.

The experiments did not lead to any very definite conclusions, but they



FIGS. 4 and 5. Seedlings of watercress (*Nasturtium officinale*). Longitudinal sections through leaf axils. Meristems are shown hatched.

did show two points of some importance. The first point was that in many sections in addition to the external meristem there was also an internal one (see Fig. 1). The internal meristem is above the cotyledon axil and appears to grow into a root. A re-examination of the section shown in Fig. 12 of Howard (1938), see also Fig. 3 of this paper, showed almost certainly that the root in that section had not originated from an internal meristem.

In one slide, in addition to the main axillary meristem producing the axillary bud, there were seen extra external meristems between the main stem and the axillary bud. Fig. 2 shows such a section. It seems very likely that such additional external meristems can become roots under the action of auxin. This may be the true interpretation of the case reported in 1938 and not the explanation then given that a leaf initial had been converted into a root. It will be noticed from Figs. 1-3 that Fig. 3 (= Fig. 12 of Howard, 1938) is of a rather old stage in the development of an axillary bud and it is probably this which makes the root look like a transformed leaf initial.

The leaf axils of watercress, *Nasturtium officinale*, are also of interest when considering the growth of axillary meristems. In watercress (see Figs. 4-5) there is normally produced in the axils of the leaves both an axillary bud and a number of roots. The roots are formed above the bud

and arise exogenously. They are thus similar in position and perhaps in origin to the induced root in the kale seedling shown in Fig. 2. According to Priestley and Swingle (1929, p. 78), such exogenous origin of roots is confined to the Cruciferae.

It therefore seems that there is no reason to suggest as in Howard (1938) that auxin can convert a leaf initial into a root. The conversion of a leaf initial into a root would be an exceptional result. Thus Thimann and Skoog (1934) found that heteroauxin inhibits the growth of axillary buds, but they did not observe any conversion of parts of buds into roots. Also Snow and Snow (1937) did not observe any conversion of shoot initials into roots when they treated the shoot apices of plants with heteroauxin pastes. The conclusion must be that meristems capable of being modified into either roots or shoots must be very small and undifferentiated.

#### 4. GROWTH OF ADVENTITIOUS MERISTEMS

As was described by Howard (1938), the auxin induced calluses of kale first produce roots and then shoots. Then when the shoots have several large leaves (and it is supposed that the shoots supply auxin to the callus) there are produced a second batch of adventitious roots. When the shoots are removed as cuttings a second batch of adventitious shoots are formed and as these grow large a third batch of adventitious roots. This cycle can be repeated several times.

It seemed possible that sections of these calluses producing both roots and shoots might help in deciding how large and how far organized a meristem has to be before it is definitely a root or a shoot. Three plants producing either their fifth or sixth batch of adventitious roots were used for the experiment. All the adventitious shoots were removed and samples of the calluses were fixed after one, two, and three weeks.

It was noticed first that the roots soon shrivelled up. This was not entirely due to the dryness of the air surrounding them as it also happened when the plants were covered by bell-jars. If the calluses have leafy shoots and the plants are covered with bell-jars, then the adventitious roots grow to several inches long. The leafy shoots may be necessary for supplying growth substances to make the roots grow.

The sections through the calluses were difficult to interpret satisfactorily. There were seen some meristems which it was impossible to recognize as either shoots or roots. The sections also showed that roots and shoots arise very close together in the callus tissue. This was also quite obvious from surface views of the calluses. Goldberg (1938) has also described the calluses induced by heteroauxin treatment of *B. oleracea* plants, and she also finds that shoots and roots can originate at the same level in the stems.

It also appeared that sections of such old calluses would never be of much use in determining the size a meristem has to be before it is definitely a

shoot or a root. A better way of attacking this problem might be the examination of young calluses.

#### 5. DISCUSSION—THE PROBLEM OF PLANT MERISTEMS

Sachs (1880-2) postulated over fifty years ago that there are two moving currents of formative substances in plants, shoot-forming materials moving towards the stem apex and root-forming substances towards the roots. The rhyzocaline, caulocaline, and phylocaline of Went (1938) are a similar postulate to that of Sachs. The conclusions of Fischnich (1938), Stoughton and Plant (1938), and Howard (1938), on the contrary, suggest that the movement of a single substance, auxin, always away from the shoot apex and towards the roots is the important factor in the differentiation of meristems into either roots or shoots. Meristems develop into shoots where the auxin concentration is low and into roots where the concentration is relatively high.

It is also interesting in this connexion to note that the cultures of proliferating procambial calluses of *Nicotiana* grown by White (1939) form buds and not roots when transferred to a liquid medium. This is to be expected since the concentration of auxin in such cultures must be very low.

While one can agree with Priestley and Swingle (1929) that it is rather too easy to postulate hormones to explain differentiation in plants and then not to proceed any further with the isolation of the hormones, &c., one can also point out that the concept of Priestley and Swingle which suggests different 'Anlagen' for roots and shoots is not a solution of the problem since it creates the new problem of what determines one type of 'Anlage' rather than the other. It does seem that it may be different concentrations of auxin which determine the type of 'Anlage' formed.

#### SUMMARY

1. The report of Howard (1938) that a leaf initial had been converted into a root meristem by the action of heteroauxin is almost certainly wrong. The true explanation would seem to be that an extra external meristem had been formed in the cotyledon axil above the axillary bud and this meristem had grown into a root.

2. The problem of the two types of plant meristems is discussed. It is concluded that root *Anlagen* are formed where the concentration of auxin is high and that shoot *Anlagen* are formed where the concentration is relatively low.

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# Observations on the Relation of Respiration to Amino Acid Supply in *Elodea*<sup>1</sup>

BY

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With five Figures in the Text

## INTRODUCTION

THE effect of amino acids on the respiration of plants has been investigated by numerous workers (Spoehr and McGee, 1923; Genevois, 1927; Schwabe, 1932). Schwabe's work indicated that when small amounts of amino acid were added to the culture medium in which *Elodea* and other aquatic plants were immersed a considerable increase in the rate of respiration resulted. The present investigation was undertaken to extend this work by determining the rate of oxygen uptake and carbon dioxide output as well as the rate of absorption of amino acids. It was hoped to employ material cultured in a number of different nutrient solutions known to affect the level of respirable material in the plant, but the results obtained in the first part of the work caused this plan to be drastically modified. The data here presented comprise three series of experiments; in the first, the diurnal fluctuations of respiration were studied in shoots grown in tap-water to show the normal trend of respiration, as without such knowledge the effects of amino acid could not be interpreted. In the second series of experiments the effects of selected amino acids on the respiration was assessed by measurement of oxygen uptake, while in the final series more elaborate methods were employed, carbon dioxide output being determined as well as oxygen uptake.

## METHODS OF INVESTIGATION

*Elodea densa* was used throughout the work. It proved to be preferable to *E. canadensis* not merely on account of the greater size of shoot but also because it lent itself more readily to culture under artificial conditions. Stocks of the plant were grown in glass tanks, 40 cm. deep, filled with tap-water which was constantly aerated and circulated. The bases of the shoots were planted in soil in small earthenware pots placed on the bottom of the tanks. During the winter the temperature of the greenhouse where the material was grown was controlled thermostatically at 18° C. and a 1,000-watt electric lamp placed

<sup>1</sup> This investigation was carried out during tenure of a Ministry of Agriculture Research Scholarship.

some 6 ft. above the tanks was used as an additional source of light to increase the day-length to sixteen hours. Under these conditions the cultures grew satisfactorily throughout the year. It may be noted that the material developed better when light was prevented from entering through the side walls of the tanks, which were accordingly covered with black paper. The growth of

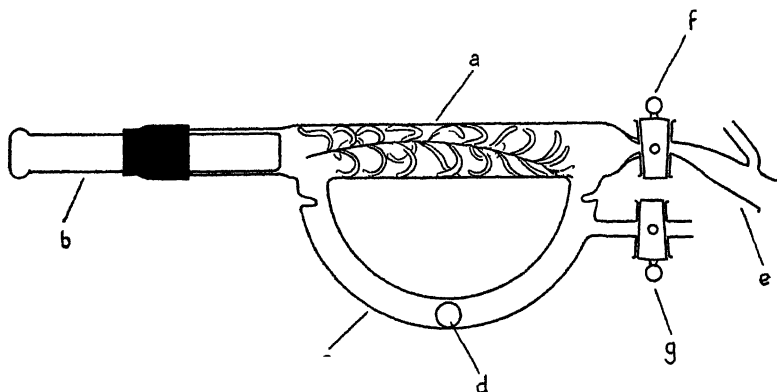


FIG. 1. Type of chamber used for experiments of short duration. For details see text

algae was satisfactorily controlled by introducing a few water snails into each tank.

Attempts were made to grow shoots in various culture solutions but active growth could not be ensured unless some solid substratum was present in which the plants could root. Even when the shoots were planted in washed quartz sand their growth did not compare with that of plants rooted in soil.

### *Respiration chambers.*

The essential requirements in respiration chambers for work of this type are as follows: (i) since respiration is measured by observing the changes of dissolved gas (either oxygen or carbon dioxide) in the medium it is necessary that samples can be removed for analysis without contact with air; (ii) the chamber must be completely filled with liquid so that no gaseous interchange occurs; (iii) there must be an efficient stirring device to eliminate diffusion gradients. Stirring by motor-driven paddles is unsuitable as they necessitate gas-tight sleeves for the driving rods, and such arrangements are cumbersome. The method was therefore adopted of rocking the respiration chamber itself and stirring by means of a free moving glass ball. Two types of chamber were employed and in both the efficiency of the stirring was proved by noting the time necessary for the complete dispersal of small drops of dye solutions added at one point.

This chamber, shown in Fig. 1, was devised to reduce to the limit the volume of culture solution. Thus large changes in the concentration of the dissolved gases were ensured so that short-period observations could be made.

Furthermore, to enable the progressive depletion of the dissolved oxygen to be followed, some device was necessary to enable the volume of the chamber to be decreased after an aliquot of liquid had been removed for analysis, so that the chamber always remained completely filled without air spaces.

The body of the chamber consisted of a wide cylindrical glass tube (*a*, Fig. 1) one end of which was fitted with a wide glass plunger (*b*), accurately ground. To prevent any leakage a rubber sleeve protected the ground glass joint. A semicircular glass tube (*c*) was attached laterally to the chamber and contained a free glass ball (*d*). Two glass taps (*f*, *g*) were attached in the positions shown, one of them (*f*) being provided with a small outer cup (*e*) fitted with a side arm.

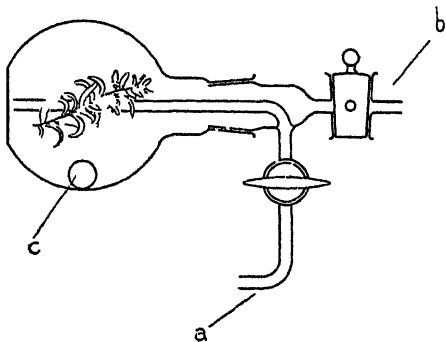


FIG. 2. Type of chamber used for experiments of longer duration. For details see text.

The chamber was used in the following way. The plunger was removed and a suitable shoot introduced into the tube; 100 c.c. culture solution, in which the dissolved oxygen was in equilibrium with air, was then run in and the plunger inserted. The chamber was then inverted and any air remaining was expelled through tap (*f*) by pressing the plunger. The apparatus was then placed in a thermostat and attached to a rocking device in such position that the ball rolled to and fro in the canal, thus stirring the liquid. To extract a sample the chamber was clamped with tube (*e*) vertical and the canal uppermost. A stream of  $O_2$ -free nitrogen was run through the outer cup (*e*) through the attached side tube, and another nitrogen lead was attached to tap (*g*). By this means, both taps being open, liquid was displaced into the cup from which a 2 c.c. sample was removed in a syringe pipette for analysis. Any liquid remaining in the cup was then forced back into the chamber by withdrawing the plunger, and, by changing the position of the chamber, the nitrogen was expelled through tap (*f*) in the same way. In this way several samples could be taken from the chamber without refilling and, since exactly 2.00 c.c. were removed on each occasion the volume of liquid remaining in the chamber at any time was known.

A different type of chamber was used in long-period experiments for which, to prevent undue depletion of the dissolved oxygen, a larger volume of liquid was required. In these experiments the chambers were refilled after each sampling. The construction of the chamber is shown in Fig. 2. At the commencement of an experiment the chamber was held vertically and filled through tube (*a*) by applying slight suction through tube (*b*). In the water bath the chamber was clamped on its side so that the ball (*c*) rolled backwards

and forwards when the rocking mechanism was set in motion. At the conclusion of the respiration period a sample of liquid was withdrawn through tube (a) into the sampling reservoir described below, oxygen-free nitrogen being meanwhile admitted into the chamber through tube (b).

*Reservoirs for sampling the liquid out of contact with air.*

In conjunction with the chamber of type shown in Fig. 2, the sampling

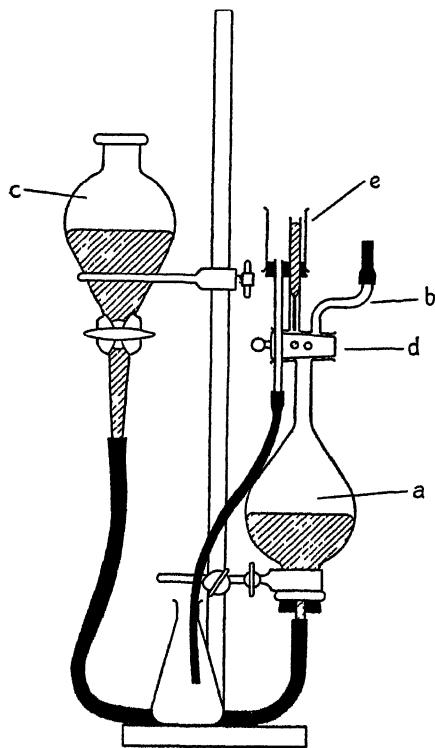


FIG. 3. Apparatus used for withdrawing fluid from the respiration chamber (Fig. 2) and sampling the same out of contact with air. For details see text.

reservoirs shown in Fig. 3 were employed. These were initially filled with mercury and the capillary side tube (b) was then connected to the vessel to be sampled. By lowering the mercury bulb (c) and turning the three-way tap (d) to connect the capillary (b) with the chamber (a) some liquid together with any air present in the tubes was drawn into the reservoir. This liquid and air, together with a little mercury, was then expelled into the small cylindrical cup (e) by raising the mercury reservoir and turning the tap (d) through 180°. No air now remained in the system, and by lowering the mercury reservoir and turning the tap (d) back to its original position the chamber (a) was filled with the liquid to be analysed. To take a sample for analysis the liquid was allowed to flow out through the cup (e) and overflow slowly. The tip of the pipette in which the sample was collected was then plunged into the cup and filled, the flow of liquid out of the chamber (a) being meanwhile regulated so that it continued to overflow slowly. The possibility of the sample being contaminated by contact with air was thus obviated. A jacket was provided round the cup to collect the overflowing liquid.

*Analytical methods.*

Dissolved oxygen was determined by the Winkler method, using syringe pipettes (see Krogh, 1935). These convenient instruments were obtained in two sizes, to hold 2 or 10 c.c. The small pipettes were used with the respira-

tion chambers of type in Fig. 1 and the larger with chambers of type shown in Fig. 2.

Dissolved carbon dioxide was estimated by the Van Slyke method (Peters and Van Slyke, 1932), using the apparatus as modified by Fuchs and supplied by Messrs. Baird and Tatlock. A mercury pipette was constructed having a capacity of about 15 c.c., the tip being fitted with a ground glass socket which filled the ground cone in the cup above the extraction chamber of the modified Van Slyke apparatus.

## EXPERIMENTAL RESULTS

### *Series I.*

In this series of experiments shoots of *Elodea* about 10 cm. long were placed in tap-water in the light for an initial period of sixteen hours at 20° C. They were then transferred to the small respiration chambers (Fig. 1), filled with tap-water which had been allowed to come into equilibrium with air at 20° C. The chambers were then immersed in a thermostatically controlled water-bath at the same temperature and the chambers were rocked in the manner already described. Samples were withdrawn for analysis every four or six hours over a period of sixty hours and the respiration values are shown in Fig. 4. At intervals the culture solution was renewed, and these occasions are indicated by arrows on the graphs. Where no arrows are marked on the curves the solution was renewed after each determination. In experiment I, three comparable shoots were employed and one oxygen determination only was made at each sampling. It will be seen from the diagram that fairly regular twelve-hourly fluctuations occurred in the rate of oxygen consumption and that the general level gradually decreased with time. In the majority of cases the peak values occurred in the periods immediately following the refilling of the chambers; this suggested that there was a close relationship between respiration rate and the oxygen tension of the water. To determine whether this was the case further experiments (expts. II and III) were carried out, two chambers being used in each case. One chamber (*b*, on the diagram) was refilled after each sampling and the oxygen determinations were replicated. The other chamber (*a*) in each experiment was refilled periodically, as shown by the arrows, and the oxygen determinations were not replicated. It will be seen that the fluctuations were not altogether eliminated by the regular refilling of the chambers. This was borne out by Experiment IV in which three chambers were employed and refilled after each sampling. The determinations were all duplicated, although, to avoid confusion on the diagrams, the individual points for one series of replicates are omitted.

The fluctuations cannot be attributed to errors in oxygen determination for the following reasons: (i) in experiment I, where single determinations were made on replicated shoots, the correlation of the values for the three replicates is clearly high, (ii) in the experiments where duplicate determinations

were made the agreement between the replicates is in general good. From these experiments it appears that somewhat irregular variations in respiration occur sporadically, but in addition that the rate of respiration is partly dependent on the oxygen tension of the water. No further work was under-

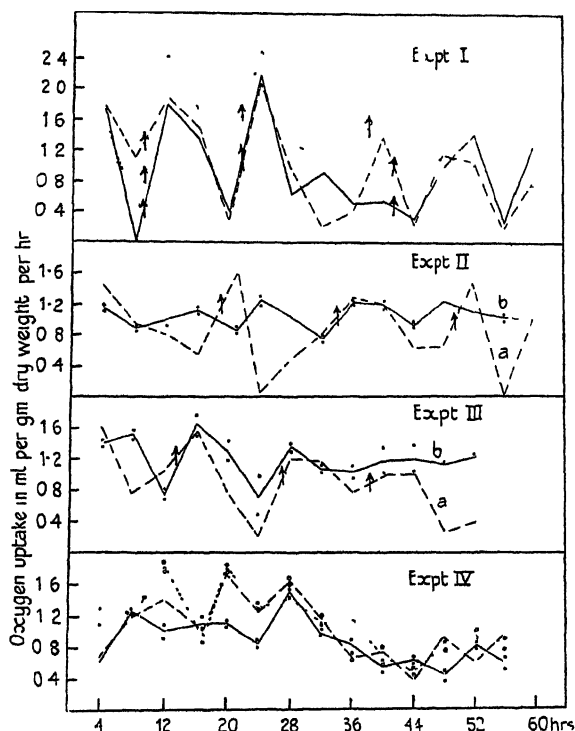


FIG. 4. Respiration changes with *Elodia densa*. Exp. I. Three replicated experiments. Single determinations of  $O_2$  at each sample. Solutions renewed at times shown by arrows. Exps. II, III. Two parallel experiments; in (a) the solution was renewed periodically as shown by the arrows and single  $O_2$  determinations made; in (b) the solution was renewed after each sample and  $O_2$  determinations duplicated as shown. Exp. IV. Three replicated experiments. Solutions changed after each sample; all  $O_2$  determinations duplicated. To avoid confusion in one series mean  $O_2$  values alone shown.

taken to show the cause of the changes in the rate of oxygen uptake. Gessner (1937) has shown that respiration rate falls rapidly when the  $O_2$  content of the solution falls below twenty per cent. saturation. He also draws attention to the importance of the oxygen tension in the intercellular spaces.

### Series II.

In view of the fluctuations in oxygen uptake in approximately twelve-hourly cycles it was decided to employ twenty-four-hour periods to determine the effect of amino acids on respiration. Chambers of the type shown in Fig. 2 were employed and the pre-treatment of the material was similar to that

in the earlier experiments. Tap-water was again used as the culture medium and the chambers were refilled every twenty-four hours when replicated determinations of oxygen uptake were made. In each experiment one control chamber was employed, being refilled with water on each occasion. The

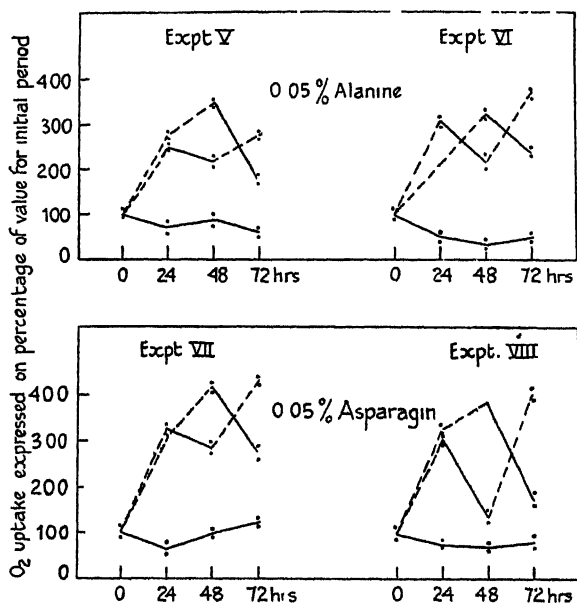


FIG. 5. Dotted lines show respiration changes (as percentage of initial value) during intervals in which amino acid was supplied; solid lines during intervals without amino acid. Replicate determinations of O<sub>2</sub> shown.

remaining chambers were filled with 0.05 per cent. amino acid for one or more periods, alanin being used in experiments V and VI and asparagin in experiments VII and VIII. In the diagrams (Fig. 5) the periods during which amino acids were employed are indicated by dotted lines. For convenience, the oxygen absorption values have been expressed as percentages of the first measurement for each shoot which has been taken as 100. It will be seen that in all cases the addition of amino acids resulted in a large increase in oxygen consumption and that this continued as long as forty-eight hours. On replacing the amino acid by tap-water the rate of uptake fell, while on again adding amino acid the oxygen consumption was once more increased. The control shoots, on the other hand, showed only small changes in the rate of oxygen consumption. These results are in agreement with those of Schwabe (1932) and the apparent increases in respiration were of the same order of magnitude, namely 200–300 per cent.

A range of other amino acids was used and similar effects in varying degrees were obtained.



*Series III.*

From the results of series II summarized above it would appear that the respiration of *Elodea* was greatly increased by the presence of amino acids, and to provide more extensive data more elaborate experiments were designed. The production of carbon dioxide was measured as well as the oxygen consumption and a dilute culture solution, buffered at pH 7.0, was used in place of tap-water.

During preliminary work it was observed that the medium containing amino acids was very favourable for the growth of bacteria; and indeed that the oxygen consumption of solutions naturally contaminated approached values comparable with those observed in the earlier experiments with *Elodea*.

This observation raised very serious doubts as to the validity of the earlier experiments as well as Schwabe's results. It was clearly essential that bacteria should be excluded if trustworthy results were to be obtained. Numerous attempts were made to obtain sterile culture conditions but no method for the sterilization of the *Elodea* plants proved successful as injury always occurred, varying concentrations of hypochlorite being tried without satisfactory results. The procedure was therefore adopted of washing the shoots several times in sterile water but, even so, complete sterility could not be ensured and to reduce to a minimum errors from this source the length of the respiration period was reduced to five hours. The results of three experiments of this type, Nos. IX–XI are given in Tables I–III respectively. In all experiments the oxygen estimates were replicated and in one experiment the carbon

TABLE I

*Experiment IX: The effect of 0.05 per cent. Asparagin on the Respiration of Elodea*

(Rates of oxygen uptake and carbon dioxide production per hour are expressed as percentages of the mean oxygen uptake for the initial period.)

Shoot I: control. Shoots II and III: experimental, with asparagin added in the periods for which the values are in heavy type.

Shoot	I.			II.			III.		
	Hours.	CO <sub>2</sub> .	O <sub>2</sub> . CO <sub>2</sub> /O <sub>2</sub> .	CO <sub>2</sub> .	O <sub>2</sub> . CO <sub>2</sub> /O <sub>2</sub> .	CO <sub>2</sub> .	CO <sub>2</sub> .	O <sub>2</sub> . CO <sub>2</sub> /O <sub>2</sub> .	
			98		104			94	
0-6	39	102	0.39	49	96	0.49	36	106	0.36
		136			123			149	
6-10	46	139	0.33	69	170	0.47	52	129	0.37
		136			130			85	
10-14	35	160	0.27	90	155	0.63	80	107	0.83
		116			157			180	
14-18	14	128	0.11	32	154	0.21	99	190	0.54
		119			136			155	
18-22	34	133	0.27	32	137	0.23	35	191	0.21
		120			119			143	
22-26	23	118	0.19	58	116	0.49	76	131	0.55

TABLE II

*Experiment X: The effect of 0.05 per cent. Asparagin on the Respiration of Elodea*

(Rates of oxygen uptake and carbon dioxide production per hour are expressed as percentages of the mean oxygen uptake for the initial period.)

Shoot I: control (except in final period). Shoots II and III: experimental, with asparagin added in the periods for which the values are in heavy type.

Shoot	I.			II.			III.		
Hours.	CO <sub>2</sub> .	O <sub>2</sub> .	CO <sub>2</sub> /O <sub>2</sub> .	CO <sub>2</sub> .	O <sub>2</sub> .	CO <sub>2</sub> /O <sub>2</sub> .	CO <sub>2</sub> .	O <sub>2</sub> .	CO <sub>2</sub> /O <sub>2</sub> .
		99			96			99	
0-8	105	101	1.05	72	104	0.72	133	101	1.33
		112			113			113	
8-12	46	100	0.41	60	111	0.54	53	118	0.46
		98			115			111	
12-16	55	99	0.54	67	107	0.60	73	113	0.65
		114			106			122	
16-20	90	114	0.79	90	114	0.82	111	135	0.86
		106			119				
20-24	71	117	0.65	62	122	0.52	63	138	0.46

TABLE III

*Experiment XI: The effect of Alanin (0.05 per cent.) on the Respiration of Elodea*

(Rates of oxygen uptake and carbon dioxide production per hour are expressed as percentages of the mean oxygen uptake for the initial period.)

Shoot I: control. Shoots II and III: experimental with alanin added in the periods for which the values are in heavy type.

Shoot	I.			II.			III.		
Hours.	CO <sub>2</sub> .	O <sub>2</sub> .	CO <sub>2</sub> /O <sub>2</sub> .	CO <sub>2</sub> .	O <sub>2</sub> .	CO <sub>2</sub> /O <sub>2</sub> .	CO <sub>2</sub> .	O <sub>2</sub> .	CO <sub>2</sub> /O <sub>2</sub> .
	32	103		26	103		28	99	
0-5	27	97	0.30	33	97	0.30	27	101	0.28
		74			87		18	76	
5-10	31	70	0.43	41	77	0.50	14	65	0.23
	27	55		21	90		18	83	
10-15	23	56	0.45	18	80	0.24	17	77	0.22
	17	51		17	89		15	73	
15-20	15	48	0.32	16	96	0.18	19	79	0.22
	13	43		25	91		18		
20-25	18	36	0.30	26	114	0.25	21	66	0.30

dioxide measurements also duplicated. The mean volume of oxygen taken up in the first period has again been taken as 100 for each shoot, the subsequent oxygen consumption and carbon dioxide production being expressed as percentages of this amount. On account of irregularities in the length of the respiration periods the results have been presented on an hourly basis. The respiratory quotients are also shown.

Periods during which amino acid solutions were employed have been indicated in heavy type.

It will be seen that the large increases in oxygen consumption occasioned

by the presence of amino acids in series II were no longer apparent. Sometimes oxygen uptake was slightly depressed when amino acids were added, while in those cases where an increase was shown it was of no great magnitude, the greatest increases being in the later periods. Bacterial contamination, due, no doubt, to bacteria adhering to the plants, was found especially towards the end of the experiments, and for this reason the present data do not provide any definite evidence that amino acids increase respiration. It will be noted that the respiratory quotient was almost always very low. This may be due to the carbon dioxide being absorbed on the fronds in the form of calcium carbonate as Arens (1936) has suggested.

#### DISCUSSION

The experiments of series II showed the large increase in oxygen consumption due to amino acid which was expected on the basis of Schwabe's work. However, in the third series, where care was taken to reduce to a minimum the contamination of the medium by bacteria attached to the plant, the effect was absent or comparatively small. No direct evidence of the presence of bacteria was sought in the second series of experiments, as at that time this source of error had not been recognized, but their presence may be presumed. Water containing a small concentration of amino acid and mineral salts is an almost ideal culture medium for bacteria and the rate of oxygen loss from such solutions has been shown to be considerable. In solutions similarly contaminated but lacking amino acids the growth of bacteria is slow and in consequence this difficulty does not arise. Hence, when bacteria are present an increase in oxygen uptake after the addition of amino acids may be due entirely to increased bacterial activity. It has been pointed out already that complete sterility was not obtained in the third series of experiments and the small increase in oxygen uptake there shown may consequently be due to bacteria. The presence of bacteria in the culture solution removed from the chambers was proved by plating out samples, but no attempt was made to evaluate the bacterial numbers and so to correct for this source of error.

From the present work it therefore appears that, if any increase in respiration results from the presence of amino acid, it is small and the large effects found both in the second series of experiments and by Schwabe are due principally, at any rate, to bacterial contamination. The fact that Schwabe found a comparatively small effect of amino acids when samples were taken after a three-hour period is in agreement with this conclusion.

It is not out of place to point out that *Elodea* and perhaps other aquatic plants are most unsuitable for work of this kind as it is difficult, if not impossible, to remove or kill the bacteria attached to their surface without damaging the living tissue.

In conclusion the author wishes to thank Professor F. G. Gregory, at whose suggestion the investigation was undertaken, for his advice and guidance throughout the work.

SUMMARY

1. Daily fluctuations in somewhat irregular twelve-hourly cycles were shown in the respiration rate of *Elodea densa*.
2. In experiments where no special effort was made to prevent bacterial contamination large increases in oxygen consumption resulted from the addition of amino acids.
3. It was not possible to eliminate bacteria entirely from the cultures but in experiments where the contamination was reduced to a minimum, the addition of amino acid caused a comparatively small increase in oxygen consumption in some cases and no increase whatsoever in others.
4. It is concluded that the large increases in oxygen consumption found by previous workers and also in the earlier experiments described here were to a large extent due to bacterial activity.

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# The Role of Growth Substances in the Regeneration of Root Cuttings

BY

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With Plate VI

## INTRODUCTION

A SHORT time ago Stoughton and Plant (1938) outlined a working hypothesis to explain the phenomena of regeneration in root cuttings of seakale (*Crambe maritima*). Since the publication of this hypothesis many other papers incorporating new discoveries and theories concerning or relating to regeneration in plants have appeared. In the present paper complete descriptions of the experiments carried out on seakale are given and a further attempt is made to elucidate the hormone theory of plant polarity in relation to root cuttings.

## EXPERIMENTAL APPROACH

Polarity has been described by Went and Thimann (1937) as the outcome of a one-way flow of auxin. As a result of this physiological mechanism auxin will tend to accumulate at the morphological base; thus a relatively higher concentration may be expected to be found at this point than at the apex of the tissue. This statement cannot be analysed too critically, but as a working hypothesis no other generalization has yet replaced it. Root cuttings ('thongs') of seakale are illustrative of polarity in its simplest form and so in examining the cause of polar regeneration, i.e. the formation of buds at the morphological apex (proximal end) and roots at the morphological base (distal end) of the thong, the concentration of growth substance is of first importance. The experiments to be described were designed to test the hypothesis that bud and root production respectively are influenced by different concentrations of growth substance.

## EXPERIMENTAL METHOD

Root cuttings of seakale were taken during the winter. The thongs were cut into lengths of 2, 4, and 8 cm., the average diameter of the thongs being 1.5 cm. The necessity for working with clean stock is emphasized since the thongs are subject to a root-rot which is fatal to their future development.

[*Annals of Botany*, N.S. Vol. IV, No. 15, July 1940.]

As a preventive measure repeated waterings with mercuric chloride (1 : 2000) were given to the compost material. Since the majority of the experiments extended over many months strict attention was paid to cultural hygiene.

The thongs were placed horizontally in either quartz sand, Cornish grit, or sterilized peat; no distinct preference is claimed for either of these materials. During 1938 thongs were grown in boxes which were placed in a glasshouse with an average day and night temperature of 20° C. Later an electric frame with thermostatic control replaced the boxes. The moisture content of the medium was maintained reasonably constant.

In order to determine the presence of growth substance the *Avena* test was used following the procedure of Went and Thimann (1937), except that the seedlings were grown in a mixture of peat and quartz sand instead of water.

### *Experiment 1.*

It was necessary in the first place to test the thong for the presence of growth substance. Sections of tissue approximately 0.5 mm. thick were cut from the thong and placed on agar blocks for a period of 1½ hours. These blocks were then cut up into smaller blocks, 2×2×1 mm., and applied to the decapitated *Avena* coleoptiles. Negative curvatures were produced in all cases. Shadow pictures were recorded ninety minutes after the application of the blocks. If the period allowed for diffusion into the agar was extended to three hours curvatures greater than the maximum which can be estimated quantitatively resulted. Further experiments are being carried out in order to determine whether it is possible to estimate quantitatively the amount of growth substance which is present at the apex and base of the thong respectively.

### *Experiment 2.*

On the hypothesis that the production of buds or of roots is determined by the concentration of growth substance the thong was treated with one of the synthetic hormones in order to raise the concentration of hormone throughout the tissues above the amount which is believed to induce buds. Thongs freshly cut at both ends to the required length were stood with 2 cm. of the apical end in a solution of  $\alpha$ -naphthalene acetic acid (0.02 per cent.). After twenty hours' treatment, at a room temperature of 18° C., the thongs were placed in quartz sand. After seventeen days the thongs were photographed (Pl. VI, Fig. 1). Roots were produced from the whole surface of the thong. Root cuttings treated with  $\beta$ -indolyl acetic acid showed a similar response except that roots were not produced from the sides of the thong. No buds were produced at the apex at the time when roots were being formed, but after a period of four weeks buds appeared in some thongs at this

point. This could be explained by the hypothesis that the applied hormone had been used up or destroyed and the cells in the apical regions were functioning under normal conditions.

### *Experiment 3.*

The converse of expt. 2 would be the production of buds instead of roots over all parts of the thong. Under normal growing conditions buds are readily formed at the apex. About a week after the appearance of buds, root initials are visible at the base of the thong. On the hypothesis already outlined a reduction in the concentration of growth substance at the base should lead to the production of buds in the place of roots. Three lengths of thongs were used, 2, 4, and 8 cm. respectively. Each group consisted of forty thongs. Every fifth day, approximately 1 mm. of tissue was removed with a razor from the apex and base of the cutting. The purpose of this method was to deplete the supply of growth substance accumulating at the base and secondly to prevent any developing buds from manufacturing fresh supplies of growth substance which would involve the transportation of unused hormone in a basipetal direction. In approximately four weeks bud initials were visible just below the apex. During the following weeks buds appeared farther down the length of the thong (Pl. VI, Fig. 2). After eight weeks buds were formed basally. Previous to the appearance of buds at the base either further root initials were produced which were in turn immediately removed or else a callus was produced which showed no further activity for 2-3 weeks. Eventually bud initials were visible arising from the callus. Thongs 2 and 4 cm. long produced buds at the base after 7-8 weeks (Pl. VI, Figs. 3, 4), whilst in the 8 cm. group buds appeared after 10-11 weeks dating from the commencement of the decapitation process. When the formation of buds at the base of the thong was considered to be probable the decapitation of the base but not of the apex was discontinued. However, when bud initials were visible at the base of the thong the decapitation of the apex was discontinued and buds were allowed to develop in this position also. Consequently, thongs with buds at both ends were obtained (Pl. VI, Fig. 5). Certain thongs which failed to produce buds at the base within 8-10 weeks but eventually responded after twelve weeks or more failed to regenerate buds at the apex. Depletion of food materials rather than of growth substance is more likely to be responsible for this lack of apical activity. Since the amount of growth substance and other substances such as food reserves probably varied for each thong no clear-cut time limits for the successive stages of development can be given and differences in the development of individual thongs were unavoidable. Approximately 75 per cent. of the thongs produced buds basally; the remaining 25 per cent. were discarded due to fungal attack. The time between the appearance, first of roots and subsequently of buds at the base,



varied with the length of the thong. No strictly proportional change over from roots to buds according to the length of the thong was noted.

#### *Experiment 4.*

A consideration of the results of expts. 2 and 3 would suggest that a thong can be induced to produce buds at the base by adopting a method of growth-substance depletion, and that subsequently the same thong could be induced to produce roots at the apex by artificially increasing the concentration of hormone at this end. Utilizing the material from expt. 3 thongs which had produced buds basally were treated with a solution of  $\beta$ -indolyl acetic acid (0.01 per cent.). The apices of the thongs were pared with a knife before being placed to a depth of 1 cm. in the solution. After 20 hours' treatment at a room temperature of 18° C. the thongs were placed in a propagating frame. Within fourteen days roots appeared from the apex (Pl. VI, Fig. 6). The addition of vitamin B<sub>1</sub> (1: 60,000) to the hormone solution resulted in the production of a larger number of emergent roots than treatment with heteroauxin alone.

Thus it is possible to reverse the normal position of roots and buds regenerating from a root cutting of seakale.

#### *Experiment 5.*

The question now arises as to the way in which the physiological behaviour and growth of the 'inverted' thong will differ from that of a normal root cutting. The 'inverted' thongs which were produced in expt. 4 were placed in pots containing coarse grit. Since this medium is chemically almost inert the plants were fed weekly with a complete nutrient solution. About twenty thongs taken from the 4 and 8 cm. groups were grown successfully for five months. The leaf growth of an 'inverted' thong (Pl. VI, Fig. 7) is shown in contrast with a normal thong (Pl. VI, Fig. 8) of the same age. Morphological differences of a fundamental nature were apparent between the two types of thong. For example, on the 'inverted' type of thong a mound of callus tissue had formed between the junction of the developing buds and the thong, (Pl. VI, Figs. 9, 10, 11). In the case of the normal thong, the base of the developing bud arose directly from its apical surface (Pl. VI, Fig. 12). The developing root systems of the two types of thong also differed. The 'inverted' thongs had subsidiary roots arising from the parent thong and also from the mound of callus tissue. Roots at the apex of the thong, which were induced primarily by heteroauxin treatment, produced a mass of fibrous roots which failed to show any sign of secondary thickening (Pl. VI, Fig. 9); on the other hand, roots arising at the base of the thong, i.e. just below the mass of callus tissue, showed secondary thickening (Pl. VI, Fig. 10). Roots arising from the mound of callus tissue also showed thickening (Pl. VI, Fig. 11). Normal thongs had

subsidiary roots, showing secondary thickening, developing only from the base (Pl. VI, Fig. 12).

#### DISCUSSION

The experimental proof of growth substance in the thong enables the analysis of the observed facts to be conducted from a hormone conception of regeneration. Since it has been shown by Went and Thimann (1937) that root production in any cutting depends on the presence of a sufficient amount of growth substance, the production of new roots at the base of the thong indicates firstly, that the required concentration is present, and secondly, that since roots are normally produced from the base only, the free-moving growth substance had accumulated at this point and thus subsequently induced roots. Priestley and Swingle (1929) showed that discs of seakale tissue 3.5 mm. thick fail to produce roots but regenerate buds over both surfaces. The total growth substance content in such a small piece of tissue is probably so low that the growth substance which accumulates at the basal surface fails to reach the level which induces root production. The longer the thong, therefore, the more probable is the formation of root initials at the distal end, since the amount of growth substance accumulating basally may be expected to be roughly proportional to the amount of tissue above the base. The results of expt. 3 involving the depletion of hormone within the thong bear out this deduction.

The use of synthetic hormones makes it possible to increase the local concentration of growth substance in the plant tissue. If the mechanism of polar transport could function more quickly the growth substance applied as in expt. 2 would presumably have been transported to the base and the normal polarity maintained. The concentration of  $\alpha$ -naphthalene acetic acid applied to the apex of the thong was very high (0.02 per cent.), but it was found that with weaker concentrations the normal polarity of the thong was not markedly disturbed. It would seem probable that a concentration of growth substance, weaker than that which must be applied to produce roots, could be found which would decrease the rate of bud formation at the apex compared with that of a normal thong. Such a concentration might be considered on some theories to produce a retarding or inhibiting effect on the rate of meristematic activity, but on the theory now being developed it is a case of alteration in the nature of differentiation rather than of change in activity. Whether this conception of inhibition can be compared with the inhibitory action exercised on lateral buds by the auxin supply of the apical bud remains to be proved. A distinction must be made between the inhibition of the differentiation of bud initials and the inhibition of growth of buds once they have been formed.

The induction of roots on the side of the thong (Pl. VI, Fig. 1) indicates that root initials may be formed at any point provided that the necessary concentration of hormone is present. The only reason, therefore, why roots

are normally produced at the distal end of the thong is that the content of growth substance, collected by virtue of the polar mechanism of the root tissue, is quantitatively suitable. The rapidity with which buds are produced apically under normal circumstances and the equal rapidity with which roots will arise in a similar position after treatment with a growth substance illustrates the association between bud and root differentiation. If a high concentration of growth substance at the distal end is due to polar transport the proximal end will contain a correspondingly less amount. Since buds arise most readily from the apex a relatively low concentration of growth substance is apparently associated with bud differentiation. It follows from this that if the distal end produces roots because it receives growth substance from regions above it the production of buds basally would be secured if only the accumulating growth substance could be removed. The results of expt. 3 bear out this suggestion. The fact that the appearance of buds began just below the apex and gradually extended downwards until buds were eventually produced from the basal callus suggests that the growth substance concentration within the tissues showed a gradient from apex to base. Again, the production of buds over the whole length of the thong indicates that no distinct preference is shown for *apical* activity with respect to bud formation once the growth substance concentration *below* the apex has been reduced to a level suitable for the initiation of buds.

Another method of producing buds basally was adopted by Neilsen Jones (1925). Thongs arranged radially with their apices outward were centrifuged for three days previous to being placed horizontally in the compost material. If by this technique growth substance is prevented from moving in a basipetal direction the production of buds at the base is in agreement with the ideas expressed in this paper. Czaja (1935), working with the dandelion, developed a similar technique to the one described in this paper for inducing the formation of buds at the base; he also found that when a paste containing growth substance was applied to the apex of a dandelion root-cutting, root initials were produced at the base as in normal cuttings where buds were allowed to develop from the apex. From these results Czaja suggested that the correlation between bud and root production is dependent on growth substances.

The response of the thongs in expt. 4 to the treatment with heteroauxin after the depletion of growth substance within the thong provides further evidence that the behaviour of meristematic tissue depends chiefly on the quantity of growth substance. Fischnich (1938) in his work on the regeneration of *Populus nigra* var. *P. pyramidalis*, suggests a similar interpretation for stem cuttings: "The formation of roots and shoots from a callus of *Populus nigra* depends upon the growth-substance content. A relatively high concentration of growth substance produces roots, whereas a low concentration induces buds. Just as a normal root-forming basal callus can be induced to produce buds so likewise can a shoot-building apex, when supplied with

growth substance in suitable amounts, produce roots.' (Translation of summary.)

Likewise, Williams (1937) working with *Selaginella* showed that the concentration of heteroauxin was a factor in determining whether an angle meristem developed as a rhizophore or as a leafy shoot. Other recent literature concerning the application of plant hormones in relatively high concentrations may be summarized as follows: Beal (1938) and Greenleaf (1937) induced bud initials, Link and Eggers (1938) caused the formation of tumour-like calluses, Goldberg (1938) and Howard (1938) produced roots followed by the appearance of buds, lastly Fischnich (1938) and Lindner (1939) induced only the formation of roots. Chouard (1938) found that in *Begonia Rex* low concentrations of heteroauxin induced buds whilst high concentrations produced only roots. It is highly probable that a plant which responds to a high concentration of hormone by producing buds could be stimulated to produce roots if a still higher concentration of growth substance was applied. So long as it is assumed that different concentrations influence the differentiation of buds and roots respectively then the actual amount may vary for any particular plant showing regeneration.

No review of the literature on this subject would be complete without a reference to Went's (1938) work on the 'calines'. As regards rhizocaline and caulocaline it does not seem that the present work excludes the presence of these hypothetical substances. If, however, these substances are present in the thong it would still appear from the above experiments that the growth-substance content, in the probable form of auxin, is the primary factor involved in cell differentiation and that such effects may be simulated, so far as root production at least is concerned, by some of the synthetic growth-substances.

In conclusion, it will be remembered that the apically induced root systems (Pl. VI, Fig. 9) of the 'inverted' thongs failed to show secondary thickening. A consideration of this feature, and also that of the abnormal mass of callus tissue which formed on the 'inverted' thongs, will be deferred to a later paper when further data has been collected.

#### SUMMARY

1. Root cuttings (thongs) of seakale were tested for the presence of growth substance by the *Avena* test. A growth substance was found to be present.
2. Thongs were treated with a solution of  $\alpha$ -naphthalene acetic acid (0.02 per cent.). Roots were produced from all points on the thong.
3. Repeated decapitation of apex and base eventually resulted in the production of buds at the base. From these, thongs with buds at both ends were produced.

4. Thongs with buds induced basally by decapitation treatments were then treated apically with a solution of  $\beta$ -indolyl acetic acid (0.01 per cent.). Roots were subsequently produced at the apex.

5. The vegetative growth of an 'inverted' thong after five months is compared with that of a 'control' thong.

6. Morphological differences between normal and 'inverted' thongs were observed. A mound of callus tissue formed on each of the 'inverted' thongs at the junction of the developing buds and parent thong. A normal thong showed no such callus.

7. Apically-induced root systems of the 'inverted' thongs showed no secondary thickening, whereas roots arising from the mass of callus tissue or from the base of the parent thong showed thickening. Normal thongs had subsidiary roots, showing thickening, arising only from the basal end.

8. The differentiation and behaviour of meristematic tissue is determined, in part, by specific concentrations of growth substance; a relatively high and low concentration of growth substance influencing root and bud production respectively.

The author wishes to express his appreciation to Professor R. H. Stoughton who suggested and directed the present investigation.

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EXPLANATION OF PLATE VI

Illustrating Mr. Plant's article on 'The Role of Growth Substances in the Regeneration of Root Cuttings'.

Fig. 1. Thongs 8 cm. long. Roots induced on all surfaces by treatment with a solution of  $\alpha$ -naphthalene acetic acid (0.02 per cent.). Apices immersed in the solution for twenty hours.

Fig. 2. Thongs 8 cm. long. Buds induced, by periodic decapitation of the apex and base, along the lateral surfaces previous to their appearance at the base of the thong. Note the gradational character of buds from the upper to the lower half of the thong.

Fig. 3. Thongs 2 cm. long. Buds induced at the base by repeated decapitation of the apex and base. Bud initials on the lateral surfaces have been removed.

Fig. 4. Thongs 5 cm. long. Buds induced at the base by repeated decapitation of the apex and base. Bud initials on the lateral surfaces have been removed.

Fig. 5. Thongs 4 cm. long. Buds developing at both ends. When buds had been induced basally, decapitation of the apex was discontinued and buds allowed to develop. Thongs placed horizontally in the compost material.

Fig. 6. 'Inverted' thongs 8 cm. long. Buds induced at the base by decapitation treatment. Roots subsequently induced at the apex by treatment with  $\beta$ -indolyl acetic acid. Apices immersed in solution (0.01 per cent.) for twenty hours.

Fig. 7. Development of 'inverted' thong 8 cm. long after five months.

Fig. 8. Development of 'control' thong after five months.

Fig. 9. Development of 'inverted' thong, 8 cm. long, after five months. A mound of callus tissue has formed at the junction of the thong and developing bud. Only fibrous roots, which were induced primarily by heteroauxin, are present at the base. (morphological apex).

Fig. 10. 'Inverted' thong, 4 cm. long, after five months. Note mound of callus and also roots showing secondary thickening arising from the parent thong.

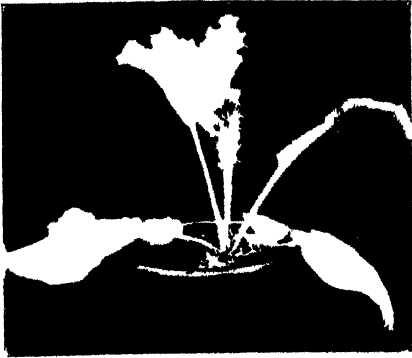
Fig. 11. 'Inverted' thong, 4 cm. long, after five months. Note root, showing secondary thickening, arising from the mass of callus tissue.

Fig. 12. 'Control' thong, 4 cm. long, after five months. Subsidiary roots arising only from base. The bud is situated directly on the apical surface of the parent thong.

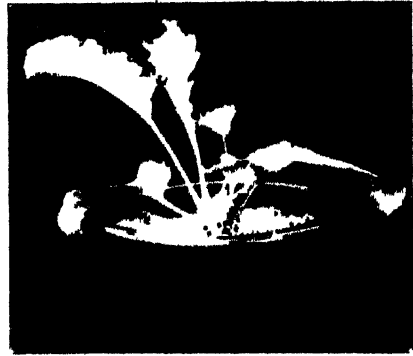


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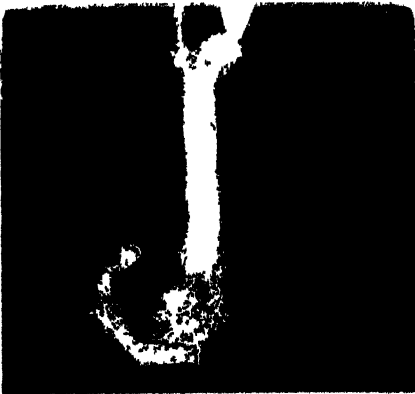




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## Studies in Flower Structure

### VI. On the Residual Vascular Tissue in the Apices of Reproductive Shoots, with Special Reference to *Lilaea* and *Amherstia*

BY

AGNES ARBER

With three Figures in the Text

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#### I. INTRODUCTION

IN recent years the validity of the comparison between a flower or an inflorescence, on the one hand, and a vegetative shoot, on the other, has been called in question. It has been stated, for instance, that the apical structure in reproductive and vegetative shoots differs fundamentally, and that reproductive shoots never possess a 'residual apex' of the type found in vegetative shoots which have come to the end of their capacity for further development (Grégoire, 1938). That such apices are actually absent in all flowers can, indeed, only be maintained by putting a strained interpretation upon admitted facts, for there are many examples of the existence of a residual floral apex comparable, to all appearance, with that of a vegetative shoot (Arber, 1937). Grégoire's general view is not, however, to be dismissed lightly, but in order to appraise his arguments we need much more exact comparative knowledge, than we at present possess, of apical structure in reproductive shoots, and in vegetative shoots of limited growth. Grégoire, in his account of the flower, confines himself to early ontogenetic phases, but for a full understanding of apical structure it is essential also to examine the stages at or approaching maturity, in which the vascular system is fully defined. The present paper is an attempt to study one small part of this field—the behaviour of the apical bundles in certain reproductive shoots in which, though there is no residual apex, the vascular tissue has been found to show special features in the apical region. The plants considered are *Lilaea subulata* Humb. et Bonpl. (Juncaginaceae), which was chosen because its spike was known to

# LILAEA SUBULATA Humb & Bonpl

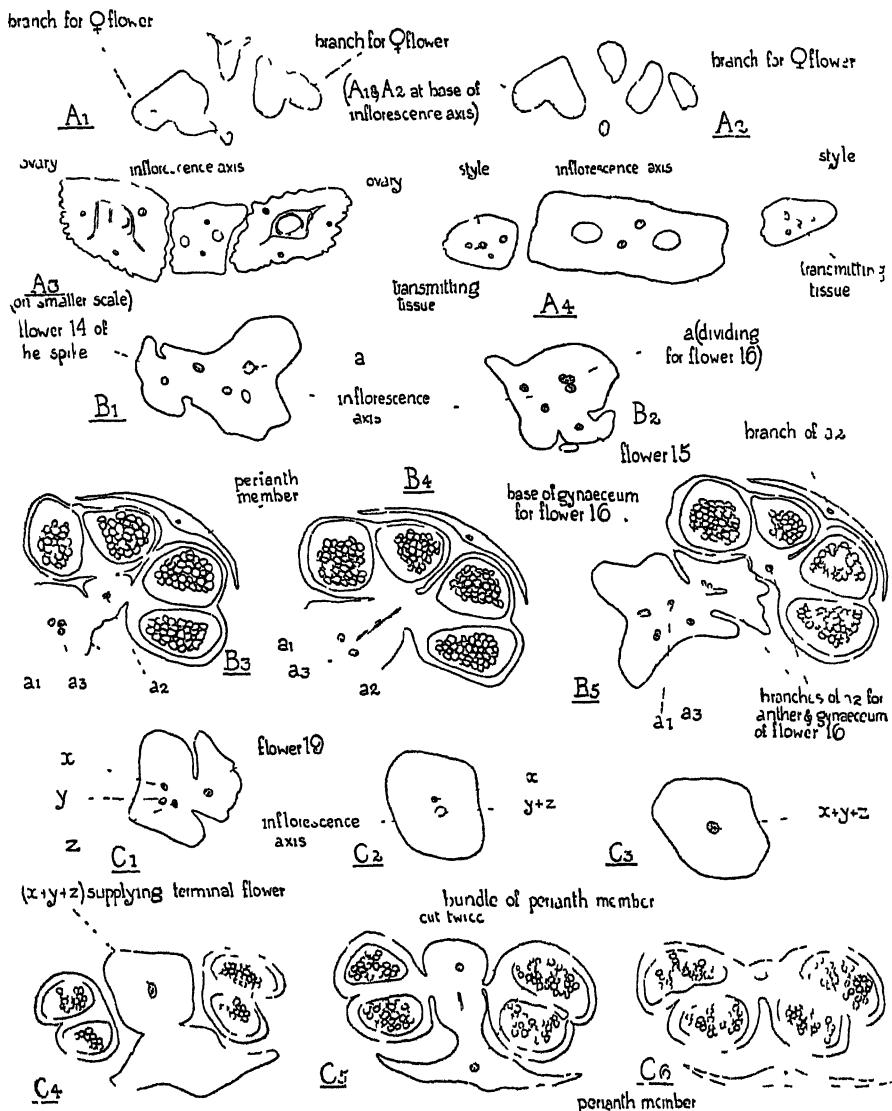


FIG. 1. *Lilaea subulata* Humb. et Bonpl. From a plant from Buenos Aires Bot. Gard., Sept. 1937. A, sections from a transverse series from below upwards through the basal region of an inflorescence. A1 and A2, bundle supply for the base of the inflorescence axis; A3, gynaeceae of the two basal female flowers, one on either side of the axis; A4, at a higher level to show the styles of the female flowers. (A1, A2, A4,  $\times 47$ ; A3,  $\times 23$ .) B1—B5, sections from a series from below upwards through the spike region of another inflorescence, passing through the origin of the 14th, 15th, and 16th flowers ( $\times 47$ ); in B3 the single collateral bundle a2 is about to pass into flower 16. C1—C6, sections from the same series as B ( $\times 47$ ), but higher in the spike, passing through the attachment of the penultimate (19th) and the ultimate (20th) flower.

have a terminal flower; and *Amherstia nobilis* Wall. (Leguminosae), chosen because Thompson (1924) recorded a peculiarity in the bundle system of the flower, which suggested that its structure might repay further study in the present connexion.

## 2. *LILAEA SUBULATA* HUMB. ET BONPL.

Through the kindness of Professor L. R. Parodi and of Mr. T. G. Tutin, I have been able to examine a plant of *Lilaea subulata* preserved in formalin, from the Buenos Aires Botanic Garden. As is well known from Hieronymus' descriptions (1878, 1889), the inflorescence of *Lilaea* bears two basal female flowers, each consisting of a long-styled gynaeceum only. Above them the peduncle is naked for some distance, and then terminates in a spike, in which one or more female flowers are succeeded by hermaphrodites, which are again followed at the apex by one or more males. A twenty-flowered spike, of which I cut serial sections, differed slightly from this description in that the flowers were all hermaphrodite, except the fifth, which was female, and the terminal one, which was male. Even in the hermaphrodite type (Fig. 1, B4, B5), which constitutes their fullest expression, the flowers are of extreme simplicity, consisting of one perianth member (sometimes interpreted as a bract), one stamen, and a gynaeceum with one ovule. The male flower consists of a perianth member (or bract) and one stamen (Fig. 1, C4–C6). In examining the anatomy I found that (in another inflorescence) two main bundles, to right and left, entered the base of the inflorescence axis, with two smaller bundles between them (Fig. 1, A1). The two main bundles, after each giving a branch to one of the two basal female flowers (Fig. 1, A2), passed up through the naked region of the peduncle (A3 and A4). In the spike of the inflorescence illustrated in Fig. 1, B and C, there were again two main bundles with two smaller bundles between them. These little bundles played only a minor part in supplying the flowers, so they will be ignored in the present brief description. In the spike, one or other of the main bundles gave a branch for each of the first nineteen flowers. An example of the mode of origin of these branches, which varied slightly from flower to flower, is illustrated in Fig. 1, B1–B4. The bundle *a* divides into three, *a*<sub>1</sub>, *a*<sub>2</sub>, and *a*<sub>3</sub>, and the lateral branches, *a*<sub>1</sub> and *a*<sub>3</sub>, continue upwards in the axis. The median branch, *a*<sub>2</sub>—a single collateral bundle—supplies a flower, the sixteenth of the spike (Fig. B4); it divides into two branches, one of which divides again for the perianth member and stamen, while the other enters the gynaeceum. When the terminal flower—the twentieth—is reached, a different scheme becomes apparent. Above the penultimate (nineteenth) flower, three bundles, *x*, *y*, and *z*, are left in the axis (Fig. 1, C1); *x* was derived from one of the main bundles of the axis, while *y* and *z* were derived from the other. In passing up, *y* and *z* first fuse (Fig. 1, C2) and then *x* unites with them (C3), so that the three strands form a single bundle, which enters the base of the male flower (C4), supplying the perianth member and the anther (C5 and C6).

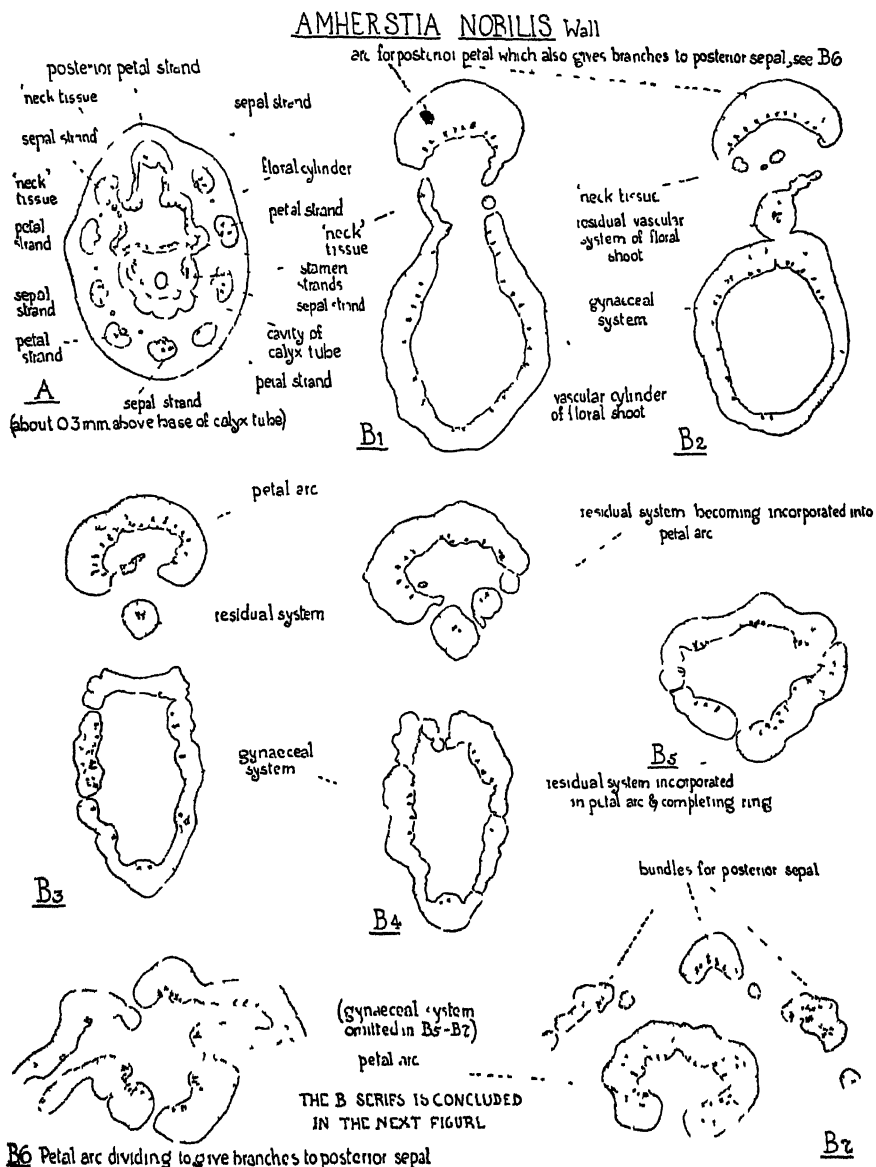


FIG. 2. *Amherstia nobilis* Wall. From flowers from 's Lands Plantentuin, Buitenzorg, 1935. A, transverse section of a flower bud about 0.3 mm. above the base of the calyx tube ( $\times 14$  circa). B1-B9 (continued in Fig. 3), sections from a transverse series through another bud, showing the history of the posterior petal system, the gynaecium system, and the vascular tissue between them. B1-B8 ( $> 47$  circa) B1, petal arc freeing itself from the floral cylinder; B2, gynaecium system detaching itself from residual system; B3, residual system isolated between gynaecium system and petal arc; B4, residual system entering petal arc; B5, residual system incorporated in petal arc; B6, branching of petal strand to give rise to sepal bundles, B7, separation of bundles for posterior sepal from petal arc. Gynaecium system omitted in B5-B9.

We thus see that whereas the first nineteen flowers receive only lateral branches from the main bundles of the inflorescence axis, the whole of the vascular tissue remaining in the axis after the penultimate flower is supplied, enters the ultimate flower. There is no residual apex to the inflorescence shoot, and no vascular tissue is left over; the flower is indeed strictly terminal to the inflorescence, incorporating into itself, as it does, the whole of the residual vascular tissue of the shoot (three bundles) instead of receiving merely a single branch bundle.

### 3. *AMHERSTIA NOBILIS* WALL.

I am much indebted to the Curator of the Herbarium, 's Lands Plantentuin, Buitenzorg, Java, for sending me in 1935 well fixed material of *Amherstia nobilis*; the following account of certain features in the anatomy is based upon serial sections through six of the flower-buds then received.

Fig. 3, B9, shows the general lay-out of the flower of *Amherstia*. There are apparently four sepals only, but the posterior segment is, actually, duplex. Wallich (1830) noted this fact in his original account of the species; he says that, in the calyx, 'laciniae 2 vexillo subjectae in unum coalitae'. The posterior petal is large, while the two front laterals are extremely small. The androecial tube is almost entirely formed from five larger and four smaller stamens. The larger stamens are each supplied by a ring of bundles, which may include twenty or more members; throughout the flower, and the bracteoles, the anatomy is complicated by this tendency to the formation of bundle rings, or concentric bundles, where simple bundles might be expected. The tenth stamen may easily escape recognition, as it has no free filament and no anther. It is limited to a minute rudiment which never becomes free from the posterior petal, and which merely completes the closure of the stamen-tube at the extreme base.

In Fig. 3, B9, the vascular tissue for all the floral parts is already differentiated and separated. From our point of view, the main interest lies in sections lower in the flower, in the region of the calyx-tube, where the connexion of the different parts of the vascular system can be observed. Fig. 2, A, which is cut about 0.3 mm. above the base of the calyx-tube of another bud, shows an outer ring consisting of five sepal and five petal bundles, while the bundles for the stamens are in process of being given off from the floral cylinder. The section is difficult to understand at first glance, because, in *Amherstia*, the continuation of the floral shoot is adnate to the posterior inner face of the calyx-tube. The posterior petal strand, and the strand for one of the sepals adjacent to it, have not, in Fig. 2, A, yet become free from the vascular ring. Each of these strands consists of an arc of vascular tissue with a neck-like connexion with the main ring; the neck is scarcely lignified, whereas the arc already has its xylem well defined. Fig. 2, B1 (from the same flower as Fig. 3, B9, which we have already described) shows the posterior petal strand at a somewhat higher level than in Fig. 2, A, and on a larger scale; it is separating

from the ring, while the 'neck' tissue is breaking up into distinct bundles. In Fig. 2, B2, the rest of the vascular system, left after the departure of the petal arc, has differentiated into a closed ring for the stipe of the gynaecium,

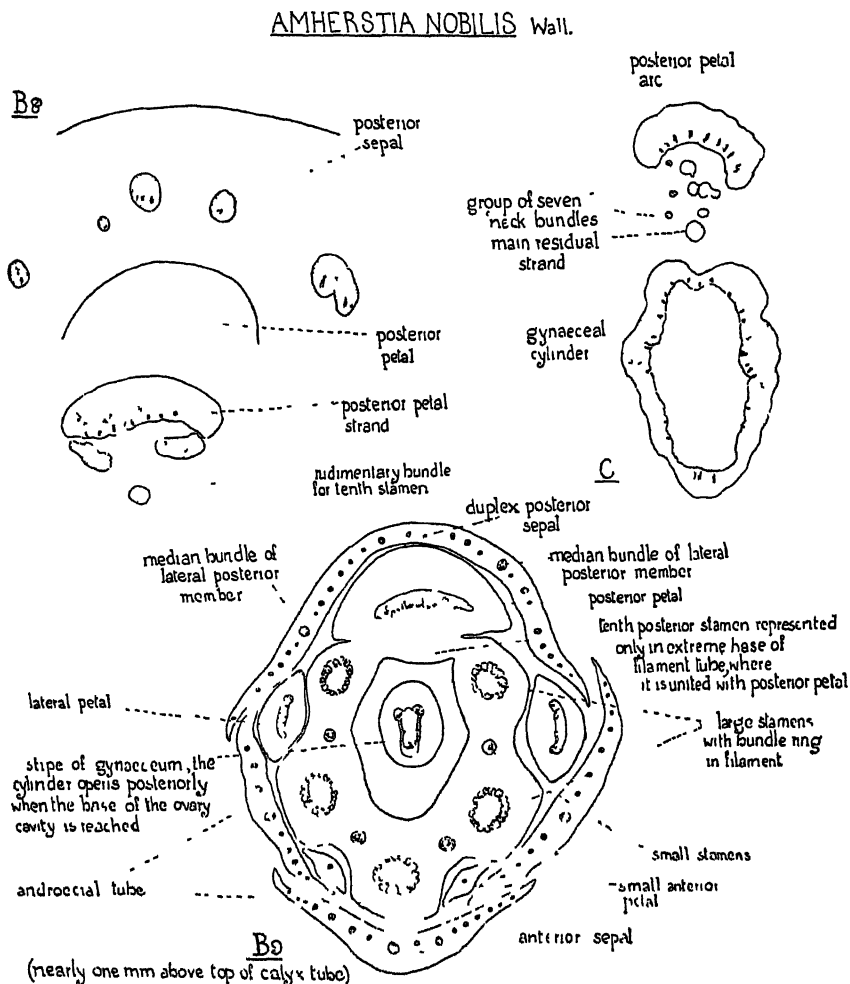


FIG. 3. *Amherstia nobilis* Wall. B8 and B9, continuation of the series in Fig. 2. B8, separation of posterior petal from posterior sepal ( $\times 47$  circa). B9, section at a higher level showing the whole flower ( $\times 14$  circa). C, gynaecium and petal systems and the bundles between them from a series through another flower, of which a section at a lower level is drawn in Fig. 2A ( $\times 47$  circa).

and a small solid strand, more or less circular in outline, with central xylem; this strand is attached to the outer, posterior face of the gynaecial ring. In Fig. 2, B3, this residual strand has freed itself from the gynaecium system, and is moving across towards the petal strand. It enters the opening of the

petal arc (B4) and attaches itself to one margin, closing the arc into a ring (B5). I have seen this residual strand, and its incorporation into the petal system, in each of the six flowers of which I have cut serial sections; the only noticeable variant among them was that in one flower the residual strand consisted at first of two bundles which subsequently fused. In the bud the structure of which is illustrated in the B series, the distance between the level at which the residual strand detached itself from the gynaeceum system, and that at which it became incorporated into the petal strand, was between 2.0 and 2.5 mm. The behaviour of the 'neck' strands is more variable than that of the residual strand. Fig. 3, C (from a different flower from the B series) shows seven 'neck' strands, one of which died out when the structure was traced upwards, while the two which were nearest to the main residual bundle, fused with it. The remaining 'neck' bundles became incorporated into the petal arc, closing it into a ring, while the main residual bundle continued its free existence further. Meanwhile the petal ring had opened again on the anterior side, and the residual bundle finally entered this gap, after an independence which had lasted for more than 3.5 mm.

In describing the B series we have reached Fig. 2, B5, in which the petal strand has attained a ring-like form by the inclusion of the residual strand. The next change is that the ring divides tangentially, and the outer part of the system contributes branches to the duplex posterior sepal (B6). In B7 the bundles destined for sepal and petal have now separated completely, and the petal strand has re-formed itself into an arc opening inwards, while in Fig. 3, B8, the boundary between the sepal and petal is visible for the first time. At about this level, a new small strand comes into view on the anterior side of the petal strand, nearly 1.5 mm. above the incorporation of the residual strand in the petal strand; in a second flower this distance was again found to be about 1.5 mm. The additional bundle is not actually a branch of the petal strand, but arises *de novo* in the ground tissue at a distance of about seven elements from the petal arc. It runs upwards for a distance of 0.02 mm. and then dies out. In another flower its length was also found to be about 0.02 mm. and it arose even nearer to the petal strand, being separated from it by four or five elements only. In a third flower its length was about 0.04 mm. In the three remaining flowers examined, no such additional strand occurred.

The natural conclusion from these observations seems to be that what I have called the main residual strand represents the apical region of the vascular system of the floral shoot, which finally loses its identity by incorporation into the posterior petal strand. The 'neck' bundles are probably best regarded as belonging primarily to the petal strand, though they may also be partially residual. The smaller strand lying within the petal strand, which occurs at a higher level (Fig. 3, B8) appears to be all that is developed of the branch for the posterior stamen. This tenth stamen is reduced almost to the point of disappearance in material described in recent years, but, as Thompson has pointed out (1924), it was evidently present in specimens of the flowers on



which early accounts were based (Wallich, 1830; Hooker, 1849); it appears, however, to have been absent in others (Anon., 1849). Thompson, in his detailed description of the flower of *Amherstia*, figured and described a 'small and independent vascular strand' radially within the posterior petal, and he identified this 'slender isolated bundle' as the 'last trace of differentiation of a vascular strand' for the undeveloped tenth stamen. I find it difficult to relate Thompson's observations exactly to my own, since he does not seem to have distinguished between the stamen strand, and what I have called the main residual strand, nor to have seen the incorporation of the latter in the petal strand. It appears that his material must have differed structurally from mine, but as he does not mention to how many flowers his description of the posterior bundle applies, it is not possible to say how general this difference was. In case the figures in the present paper should be compared with those of Thompson, it should be noted that he places his drawings so that the posterior (adaxial) face of the flower is turned towards the foot of the page. This is the reverse of the usual convention for floral diagrams and sections, in which the ground plan is represented so that the axillary shoot is set, as it is in nature, *above* the axillant leaf; it is this recognized scheme which is followed in the diagrams illustrating the present paper.

#### 4. DISCUSSION

In a former paper (1931, pp. 338–42) I have described the vascular relations of the inflorescence tip and the ultimate flowers in certain Fumarioideae. The variants met with may be summarized briefly as follows. In an inflorescence of *Corydalis nobilis* Pers., it was found that, when the strands for the two uppermost flowers had been differentiated, two shoot strands remained over, though there was no further flower for them to supply. One of these divided, and the two branches attached themselves to the strands for the two uppermost flowers; the other gave off two branches (which also divided themselves between the strands for the flowers) while it died out itself. The minute apex of the shoot was thus left wholly non-vascular. A similar condition was found in an inflorescence of *Corydalis bulbosa* Reichb. In a raceme tip of *Fumaria officinalis* L., below the ultimate flower the axis contained a single shoot bundle in addition to the normal supply for the flower. This shoot bundle divided itself between the two bundles for the flower pedicel, so that the shoot apex was again left wholly non-vascular.

We thus see that in these Fumarioideae, when the inflorescence shoot reaches a stage beyond which it can develop no further, vascular tissue, which would normally have been used for the succeeding flowers, may form a supernumerary supply for the ultimate flower or flowers, entering their pedicels and fusing with their bundles, while the apex itself is deprived of a vascular supply. In *Fumaria officinalis* I have found another variant also, in which after the penultimate flower was given off, there was only enough

vascular tissue left to supply the ultimate flower; in this example no residual apex remained between the uppermost flowers.

The situation in the Fumarioideae can be paralleled closely in the example from among the Monocotyledons—*Lilaea subulata*—described in the present paper. In the inflorescence, details from which are illustrated in Fig. 1, B and C, the ultimate flower, instead of receiving merely a lateral branch from the vascular system of the inflorescence shoot, is supplied by a composite bundle resulting from the fusion of the three strands left in the inflorescence axis above the penultimate flower. The inflorescence shows no trace of a residual apex.

These instances demonstrate that the shoot bundles left over in the tip of an inflorescence, instead of merely dying out in a residual apex, may unite with the bundles of the uppermost flowers, while the residual apex is left non-vascular, or is suppressed altogether. The example of *Amherstia*, described in the present paper, shows that a precisely parallel situation may arise when it is an individual flower, and not an inflorescence, that is in question. Here the residual vascular system of the floral shoot, left after the formation of the gynaecium system, does not die out, but, after passing upwards for two or three millimetres, becomes incorporated in the vascular system for the posterior petal. In the buds which I examined, which were all nearly mature, there was no indication of an actual residual apex to the floral shoot. We have to bear in mind, however, that Newman (1936) has demonstrated that the gynaecium of *Acacia* is lateral in origin, although at an early stage this fact ceases to be recognizable;<sup>1</sup> it thus seems reasonable to suppose that the gynaecium in some of the other Leguminosae may eventually be shown also to be lateral.

The parallelism between inflorescences and individual flowers, in the behaviour of the apical vascular system, may be summarized as follows. In certain Fumarioideae and in *Lilaea*, the residual apical system of a shoot of the first order (inflorescence) disappears by fusion with the vascular system of one of its own shoots of the second order (flower); while, in *Amherstia*, the residual vascular system of a shoot of the second order (flower) disappears by fusion with the vascular system of one of its own lateral phyllomes (petal). This comparison may be of some significance as showing that a phyllome may be related to the single shoot of which it forms a part, in the same way as a single shoot is related to the shoot complex of which it forms a part.

No comparable mode of disappearance of residual apical vascular tissue is known, so far as I am aware, in vegetative shoots. Indeed, its occurrence there is not very probable; for it is not easy to find examples of vegetative shoots in which limitation in growth is associated with extreme reduction of the shoot apex, and it is only in such shoots that bundle fusions of the type described in the present paper would be expected. If these fusions prove to

<sup>1</sup> Despite a detailed criticism by Thompson (1936), the evidence on which Newman founds his conclusions appears to be convincing.

be confined to reproductive shoots, it might perhaps be concluded that this fact affords evidence favouring Grégoire's contention that vegetative and reproductive shoots are incommensurable. I do not think, however, that this conclusion would be sound. For strict parallelism between vegetative and reproductive shoots is not incompatible with the existence of a marked distinction arising out of one of their primary differences—namely, that terminal growth is prolonged in the vegetative shoot, but suffers early inhibition in the reproductive shoot.

## 5. SUMMARY

In *Lilaea subulata* Humb. et Bonpl., residual vascular tissue in the apex of the inflorescence is found not to die out, but to disappear by fusion with the bundle supply to the apical flower. Similar instances have been previously described for inflorescences of the Fumarioideae (Arber, 1931).

In *Amherstia nobilis* Wall., the residual vascular tissue in the apex of the flower is found not to die out, but to disappear by fusion with the strand supplying the posterior petal. (In three of the six flowers examined, in the neighbourhood of this composite strand a minute bundle arose which may be interpreted as the trace for the tenth stamen.)

A comparison of the apices of these reproductive shoots thus shows that a phyllome may be related to the single shoot of which it forms a part, in the same way as a single shoot is related to the shoot complex of which it forms a part.

It is possible that the mode of disappearance of apical shoot bundles described in the present paper may prove to be confined to reproductive shoots (inflorescences and flowers); but, even if this is so, it cannot be treated as evidence supporting Grégoire's view (1938) that vegetative shoots and reproductive shoots are in no way comparable. It would merely be yet another example of the numerous secondary differences between these two types of shoot, ultimately traceable to that checking of apical growth, which so commonly supervenes in the reproductive phase.

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# Further Observations on the Morphology and Anatomy of the Flower in *Salvia*

BY

EDITH R. SAUNDERS

With ten Figures in the Text

IN an article which appeared recently (Saunders, 1934) I brought forward certain evidence furnished by late flowers of *Salvia schiedeana* showing that when floral members have apparently been lost—in this case the two postero-lateral stamens—the corresponding vascular bundles may nevertheless persist (Saunders, 1934, pp. 144-7 with Figs. 21, 22, 24). Other evidence of a similar nature adduced from Primulaceae made it clear that A. Arber's statement that 'the belief in vascular survival has been responsible for an artificial and erroneous interpretation' of the primulaceous androecium (Arber, 1933) can only be regarded as the expression of a preconceived opinion not to be shaken by facts which cannot be reconciled with it.

The present further observations on *Salvia* were undertaken in view of two circumstances, (1) that Arber (1933) by way of support to her contention that there is no evidence for the persistence of vascular bundles after morphological form has been lost cites the statement of an earlier writer, Grélot, that whenever there is complete abortion of a floral member the corresponding vascular bundle never persists, and (2) that *Salvia* was one of the genera examined by Grélot (1897) in his general survey.

It would seem from Grélot's account that he had already arrived at the above view before *Salvia* came under his notice, for he did not fail to remark the presence of the two vascular bundles in the corolla-androecium tube of this genus *occupying the same positions* as the two bundles which in other Labiatae serve the fully developed and functional postero-lateral stamens (*italics mine*). To adopt the natural and obvious interpretation of this fact, viz. that these *Salvia* bundles are the persistent bundles proper to the aborted staminal members, would be to admit that his conviction was not well founded. Some other explanation must, he concluded, be the true one, and the one he puts forward is this. Since vascular bundles do not (as he believed) persist after loss of morphological form has occurred, the bundles in question cannot be the bundles of the 'lost' postero-lateral stamens although they occupy the positions of such bundles. What then must have happened is that some strand in the reticulate network of the petal lobe must have come

into contact at some point with the now unoccupied track of the lost true stamen bundle. Finding this track unoccupied the strand in question entered it and grew down it to the bottom of the corolla tube (loc. cit., p. 130). On this conception it is evident that it would need further to be supposed that usually two such strands, one in each neighbouring petal lobe, converged at some point in this imagined track and, having met, continued not only down the whole length of the corolla-androecium tube as a single cord but on through the cortex of the axis to the central vascular cylinder.

Now this conception of the mode of origin of the bundles in question is not, it will be noted, founded on any evidence. It was clearly a supposition put forward by Grélot in order to bring his observations into line with a belief which he had already formulated.

In view of the evidence already brought forward in my preceding article, all that is necessary here in order to complete the chain of positive evidence proving that Grélot's sweeping assertion has no value is to demonstrate by means of the accompanying figures: (1) that in those *Salvia* forms in which the two postero-lateral members of the androecium attain separate morphological form but are reduced to staminodes, the two vascular bundles which run up the corolla-androecium tube on these radii terminate in these structures from the first moment of their appearance, precisely as the two bundles on the antero-lateral radii terminate in the two fertile stamens, so that it is beyond doubt that the two pairs of bundles are those proper to the two pairs of staminal members; (2) that the two postero-lateral bundles which serve the two postero-lateral stamens in Labiatae with four fertile stamens are identical with those occupying the same radii in *Salvia*.

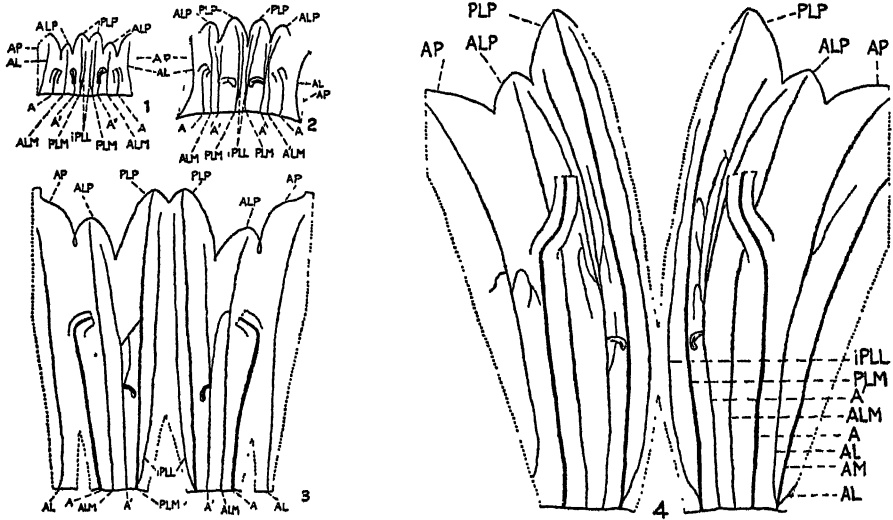
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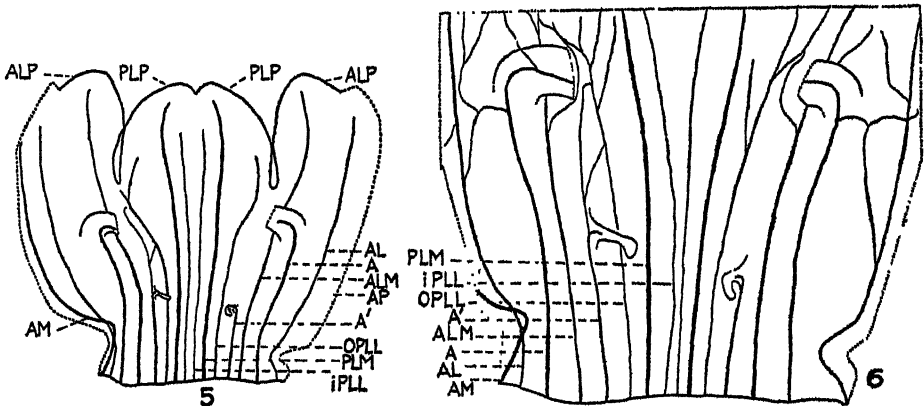
Abbreviations used in explanation of the figures:

*A*, stamen bundle; *A'*, staminode bundle; *AL*, basal lateral of the anterior petal midrib; *ALM*, antero-lateral petal midrib; *ALP*, antero-lateral petal (margin or cut edge); *AM*, anterior petal midrib; *AP*, anterior petal (margin or cut edge); *iPLL*, inner basal lateral of the postero-lateral petals; *oPLL*, outer basal lateral of the postero-lateral petals; *PLM*, postero-lateral petal midrib; *PLP*, postero-lateral petal (margin or cut edge).

In general only midrib bundles, primary laterals, and some of the finer strands originating from these bundles at or near the level at which the stamen and staminode main bundles turn horizontally are represented. In all figures the staminal filaments have been cut away close to the level at which they become free. The lip of the anterior petal has also been removed. Where the midrib of this petal lies in the line of section of the tube it is not shown.



FIGS. 1-4. *Salvia splendens*. The corolla and androecium. The tube has been split near the mid-line in front and opened out. Fig. 1. From a very young bud at the stage at which stamens and staminodes become free close to the tube base. Only the main bundles and the primary laterals of the postero-lateral petals and of the anterior petal are, as yet, differentiated. Fig. 2. The same from an older bud showing an early stage in the differentiation of a branch system from the staminode bundles. Fig. 3. The same from a still older bud showing anastomoses between the staminode systems and those of the neighbouring petals. [The base of the corolla-androecium tube has been cut at three points in order to allow the whole preparation to lie flat.] Fig. 4. The corolla and androecium of a large bud showing a later stage in the differentiation of the branch systems of the staminode bundles and the appearance of a few fine branches from the primary (basal) laterals of the anterior petal midrib. [In order to allow the preparation to lie flat it has been split in the mid-line top and bottom.]



FIGS. 5, 6. *Salvia farinacea*. Fig. 5. The corolla and androecium treated as in Fig. 1. The staminodes spring from the tube at different levels, an unusual feature. The vascular bundle of the lower one (right) remains unbranched; that of the other has given rise to a characteristic branch system. Fig. 6. A portion of the corolla-androecium tube seen in Fig. 5 more highly magnified showing some of the finer anastomosing veins derived from the main bundles of petals, stamens and staminodes in the region depicted.



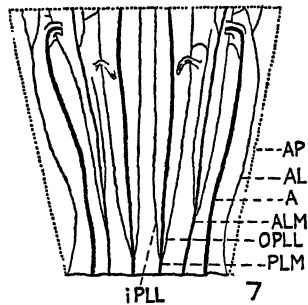
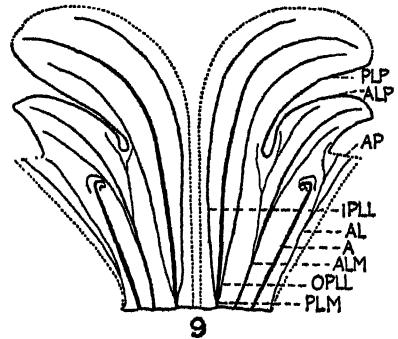
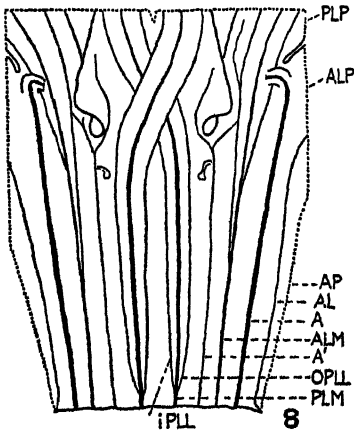


FIG. 7. *Salvia uliginosa*. The corolla-androecium tube treated as in Fig. 1. Free staminodal structures have been developed, but the two corresponding vascular bundles from the tube base up to the level at which these structures spring from the tube have been lost, only the terminal portion of the bundles traversing these structures being developed. [The process of degeneration here follows a course the reverse of that previously described in some end-of-the-season flowers of *S. schiedeana* in which it is the free staminodal structures which are absent while the remainder of the corresponding vascular systems persist (see Saunders, 1934, p. 144, Fig. 21).]



FIGS. 8, 9. *Salvia Sclarea*. Fig. 8. A portion of the corolla-androecium tube from an open flower taken in summer. The torsion of the vascular systems of the two postero-lateral petals is due to the overlapping of these petals after the upper lip of the corolla has been split in the mid-line in order to allow the preparation to lie flat. [The bottom of the cut is indicated by the nick in the outline in the mid-line at the top.] Both free staminodal structures and the corresponding vascular systems are present, but the termination of the main bundle has been lost, the free staminodal structure being without vascular tissue. Fig. 9. The corolla and androecium from an unopened bud taken in autumn and cut in half in the median plane. Both staminodes and the corresponding vascular systems are lacking.

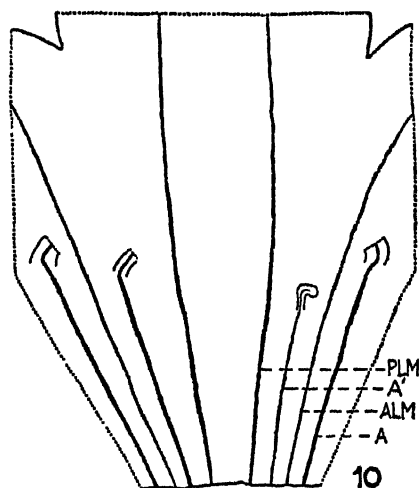


FIG. 10. *Monarda didyma*. Portion of the corolla-androecium tube from a flower in which one of the two postero-lateral members of the androecium has degenerated into a staminode while the other has retained the form of a fertile stamen.



# The Effect of Ringing on the Upward Movement of Solutes from the Root<sup>1</sup>

BY

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With four Figures in the Text

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## I. INTRODUCTION

MUCH of the evidence concerning the channel for the upward movement of soil solutes is derived from ringing experiments. Thus Curtis (1923) was first led from such experiments to doubt the current view that soil solutes ascend in the wood. His procedure was to select pairs of branches on a plant, to ring one, and after some time, to compare the nitrogen and ash contents of the two branches. He found that the leaves on a ringed branch always contained less nitrogen and ash than those on a normal or unringed branch. His experiments ran for the most part for a matter of months, and while leaves were sampled in all experiments, the stems were analysed in only one experiment, and here, too, they were found to contain less nitrogen than the controls. He omitted to find out whether the ringed branch as a whole had increased its content of nitrogen and ash, for he made no collection of stems at the time of ringing.

Clements (1930), whose experiments also continued for a matter of months, repaired this omission and demonstrated that a ringed branch was able to take up nitrogen and to increase its ash content. He sampled whole branches before

<sup>1</sup> Paper No. 21 from the Physiological Department of the Cotton Research Station, Trinidad.

and after ringing and thus was able to demonstrate that soil solutes could ascend the stem in the wood. He omitted to sample unringed branches at the end of the experiment when he sampled the ringed branches and was thus unable to confirm or refute Curtis's conclusion that ringing checked the upward movement of nitrogen and ash. He demonstrated, in short, that soil solutes ascend the stem in the wood, but did not show that they do not also ascend through the bark.

Recently, Clements and Engard (1938) have published the results of ringing experiments in which they determined the amounts of nitrogen and ash in branches before ringing and in ringed and normal branches at the end of the experiment. They ringed nine different types of plants and were able to show that the effect of ringing 'is somewhat characteristic of the species and of the environment in which the plant is growing'. The effect of the ring was greatest in *Salix* and least in *Pyrus* (apple). Table I is taken from Clements and Engard's results.

TABLE I

				Ash per shoot. (mg.)	Total nitrogen per shoot. (mg.)
<i>Pyrus</i>	1st control	.	.	107.74	20.53
	Girdled.	.	.	292.50	50.95
	2nd control	.	.	262.20	51.49
<i>Salix</i>	1st control	.	.	14.56	5.41
	Girdled.	.	.	49.13	7.51
	2nd control	.	.	362.75	55.73

They point out that girdling prevents the development of new wood and damages the old wood so that a transfer from one annual ring to another becomes obligatory. The structure of the wood, they think, is thus responsible for the different rates at which water and salts were transmitted past the region of the ring. 'It appears', they say, 'that the girdle, as a break in the continuity of the phloem, does not affect the upward movement of salts; but rather that the girdle, as it affects the movement of water, influences the upward movement of salts in the xylem.'

While Clements and Engard have demonstrated that soil solutes can ascend the stem in the xylem, they have not shown, except in one species, that they can travel in this channel in amounts as great as in the whole stem. They have made out a *prima-facie* case that the differences in the amounts of nitrogen and ash in ringed and normal branches are due to the effect of the ring on the movement of the transpiration current. On the other hand, it might be argued that the inability of most of their ringed branches to obtain their normal supply of soil solutes is due to the fact that these species utilize both bark and wood in upward movement. Moreover, if there is a downward movement of nitrogen through the bark, as Maskell and Mason (1929) claim to have demonstrated, ringing should tend to increase the nitrogen content of a ringed

branch above that in an unringed branch. Thus, there would still appear to be an element of doubt concerning the correct interpretation of such experiments, especially as neither Curtis nor Clements and Engard have demonstrated that there was uptake of soil solutes during the course of their experiments, for they only analysed leaves or branches and not whole plants.

In the present paper an experiment is reported in which branches were ringed as in the experiments of Curtis and of Clements and Engard. In this experiment the plant, apart from the root and a small section of stem, consisted of only two branches, one of which was ringed, while in the experiments of Curtis and of Clements and Engard apparently only a small proportion of the branches on the tree was ringed. The whole plant was analysed and the collection of branches was made at short intervals. A feature of the experiment is that analyses were made of both calcium and phosphorus. As calcium has always been found to be immobile in the phloem, its distribution provides an index to the distribution of the transpiration current between normal and ringed branches. By comparing the distribution of the phloem-mobile element phosphorus with that of calcium, we are thus able to assess the importance of the transpiration current and the phloem in determining the distribution of solutes between ringed and normal branches.

## II. METHODS

Sea Island cotton plants were grown in the field and when seven weeks old the apical bud was removed, causing the sub-apical buds to grow. Two of these buds from adjacent nodes were allowed to develop, the rest being removed as they began to grow. The result (see Fig. 1) was a plant with a short main stem and two branches consisting only of stem and leaves. The plants were fifteen weeks old when the experiment began and the branches approximately 60 cm. long. The branches were marked, one with yellow and the other with black wool, so that the taller branch was marked alternately yellow and black. The plants were graded on the height of the two branches and the grading checked on the diameter of the stem below the fork.

There were two treatments. In the Normal group neither of the branches was ringed. In the Ringed group a  $\frac{1}{2}$ -in. ring of bark was removed from one branch (branch A) as shown in Fig. 1. The ring was located at the base of one of the branches. The exposed wood was protected by a strip of surgical adhesive tape, followed by wax over the tape to fill in the ring. Callus was removed as it appeared. There were an initial collection of Normal plants and three subsequent collections of Ringed and Normal plants. For each treatment there were three samples at each collection and 25 plants per sample. Immediately before collection  $\frac{1}{2}$ -in. rings of bark were removed from both branches of the Normal group and from the unringed branch of the Ringed group. This bark was of course rejected. The results are expressed on the sample basis and represent dry weight and the weights of phosphorus and of

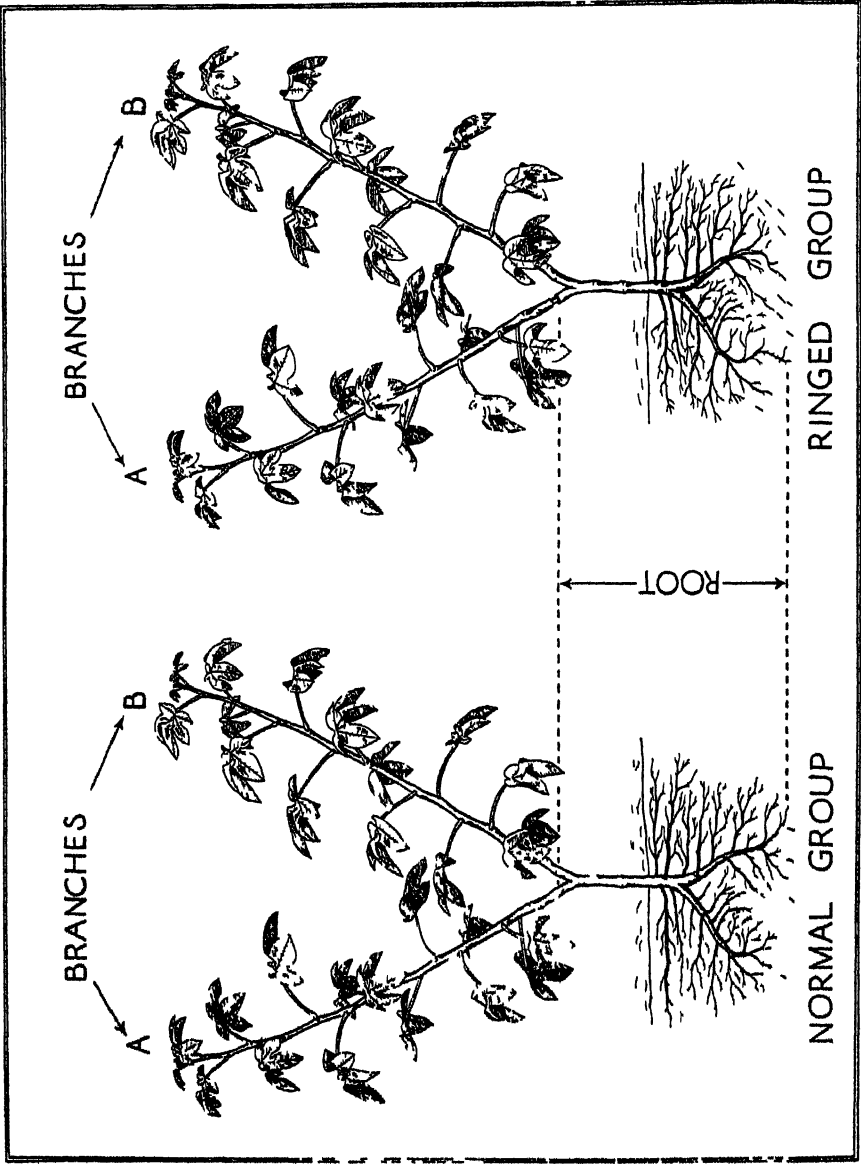


FIG. 1. Showing branching of plant and position of ring. Branch A was ringed in the Ringed group

calcium per sample of 25 plants. The following was the sequence of events:

*Time-Table*

Day	0	Ringing and initial collection of three samples of Normal plants.						
"	3	Collection of three samples of each of the Ringed and Normal plants						
"	8	"	"	"	"	"	"	"
"	14	"	"	"	"	"	"	"

Thus the whole experiment lasted for a period of only fourteen days, a period much shorter than that used by either Curtis or Clements and Engard.

### III. RESULTS

#### A. *Dry Weight.*

The changes in dry weight are shown in Fig. 2. Branch A represents the ringed branch of the Ringed group and the corresponding branch of the Normal group. Branch B represents the unringed or normal branch of the Ringed group and, of course, the corresponding branch of the Normal group (both branches being unringed). The remainder of the plant, consisting of the root, the short main stem, and the fork, is referred to as the root in the text (see Fig. 1).

It will be seen that the dry weight of the whole plant was unaffected by the ringing of one of the branches up to the 8th day of the experiment. The difference in favour of the Ringed group is partially significant on the 14th day. It will be noticed that this increase is entirely due to the ringed branch of the Ringed group (branch A).

The ringed branch (branch A) of the Ringed group exceeded the corresponding branch of the Normal group at all collections, while the unringed branch (branch B) of the Ringed group showed the reverse behaviour. Ringing also resulted in a marked decline in the dry weight of the root. The results are clearly what would be expected if carbohydrates are manufactured in the leaves and distributed through the phloem. The cessation of the supply of carbohydrate to the root from the ringed branch would steepen the sugar gradient between the unringed branch and the root, thereby producing an increase in the rate of carbohydrate movement out of the branch.

#### B. *Phosphorus.*

The results for phosphorus are shown in Fig. 3. The uptake of phosphorus by the whole plant was slightly reduced by ringing, but the reduction in uptake shows no tendency to increase during the fourteen days the experiment lasted. The reduction in uptake did not reach the level of partial significance.

The distribution of phosphorus between the three regions was very similar to that of dry weight. The ringed branch (branch A) exceeded the normal. In the other two regions (branch B and root) the Normal group exceeded the



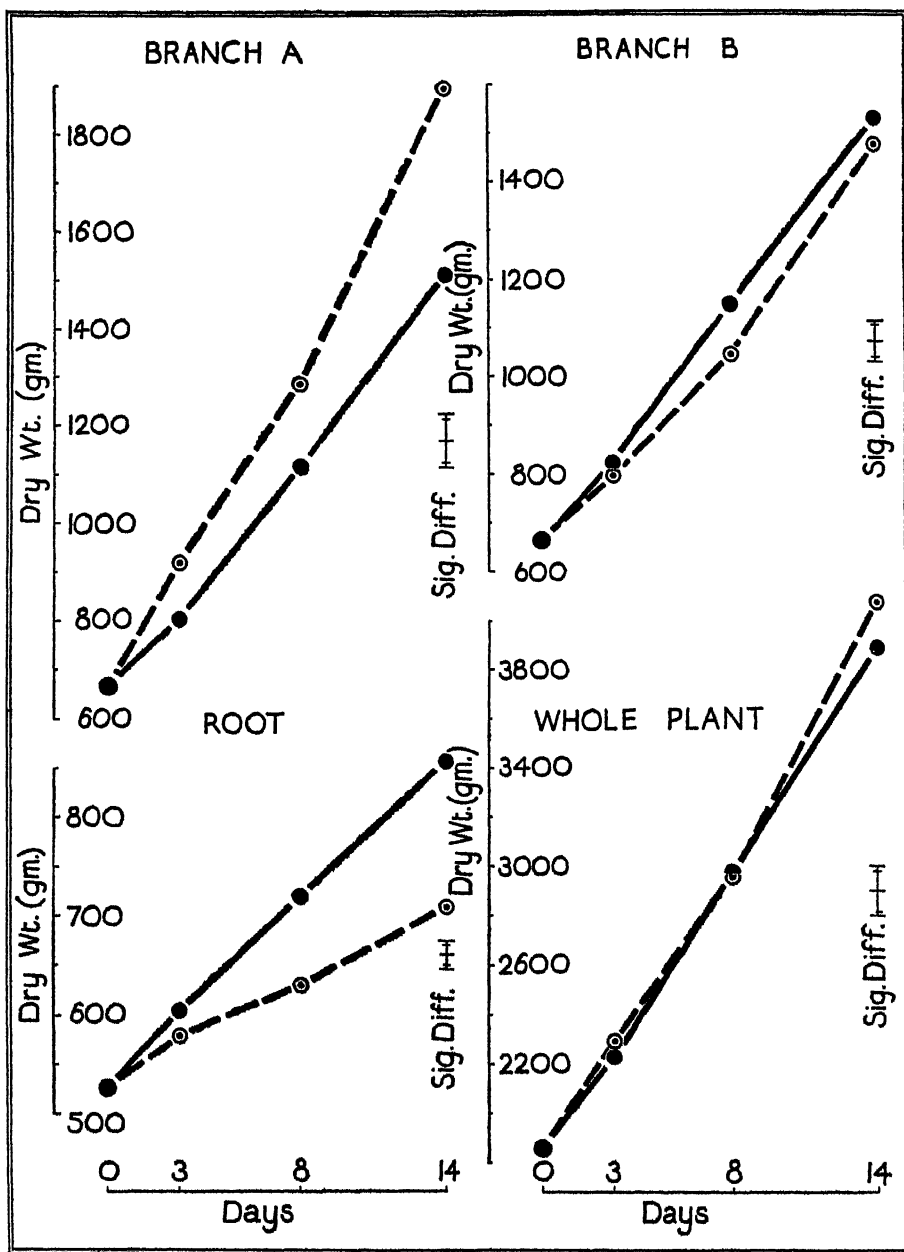


FIG. 2. Showing dry weights of whole plant and of regions per sample of 25 plants for the Ringed group (broken line) and for the Normal group (continuous line).

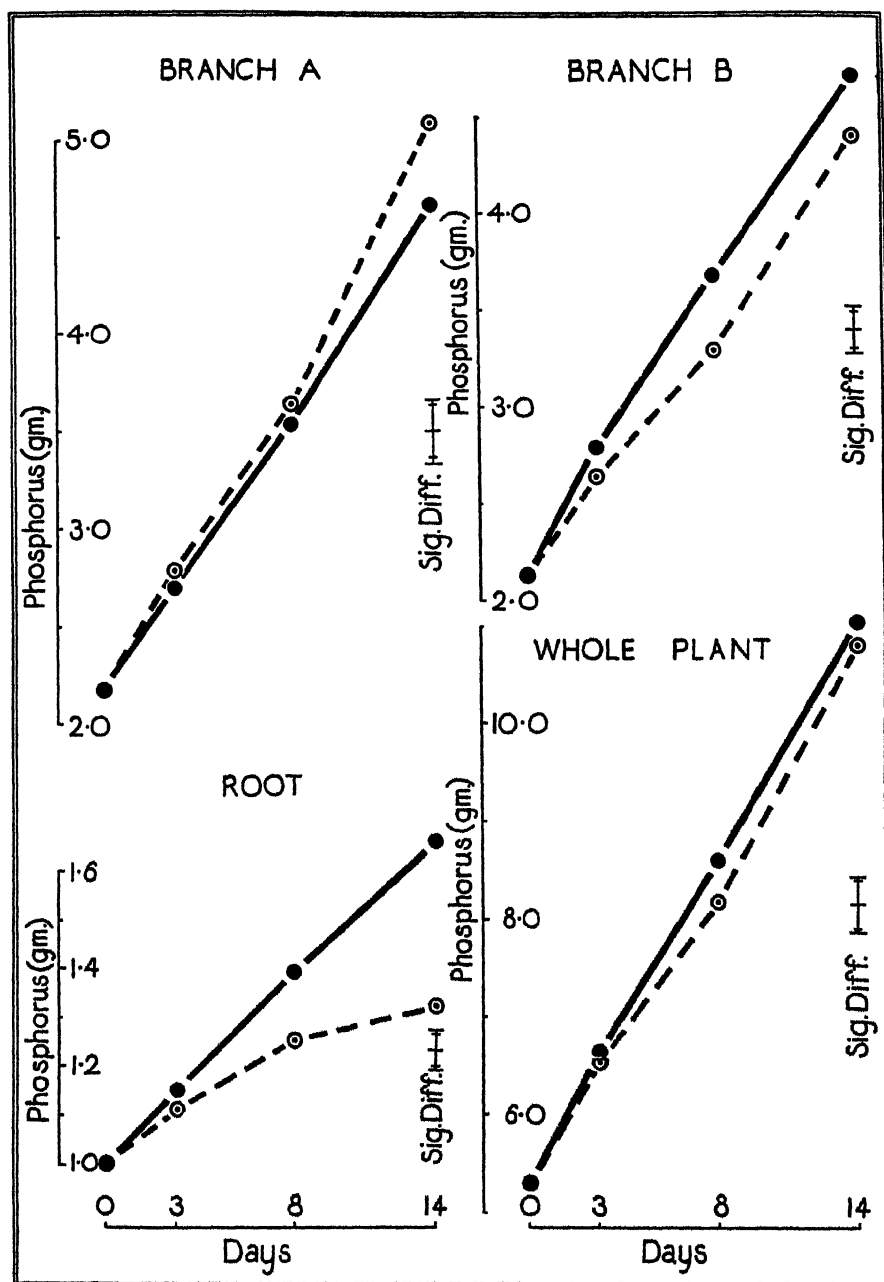


FIG. 3. Showing weights of phosphorus in whole plant and in regions per sample of 25 plants for the Ringed group (broken line) and for the Normal group (continuous line).

**Ringed.** It seems clear that phosphorus traversed the region of the ring in the wood and that on its return journey from the leaf it was trapped by the ring. In branch B and the root the reduction in the amount of phosphorus as a result of ringing was caused in part by the retention of phosphorus in branch A by the ring and in part by the reduction in uptake by the root. From the graph it is impossible to say whether more or less phosphorus entered the ringed branch than the normal, for the amount of phosphorus in the ringed branch shows only how much entered it, while the amount in the normal branch depends on how much entered and how much left it. It will be noticed that the rate at which phosphorus accumulated in the root of the Ringed group declined as the experiment proceeded.

### *C. Calcium.*

The results for calcium are shown in Fig. 4. Uptake by the whole plant was checked by ringing in much the same way as phosphorus uptake. In the ringed branch (branch A) the amount of calcium declined below that in the normal branch. In branch B there was no significant difference between the two groups. In the root the Ringed group declined below the Normal, but to a smaller extent than was the case for phosphorus. As calcium is not mobile in the phloem, its distribution between the two branches (i.e. A and B) of the Ringed group may be used as an index to the distribution of the transpiration current. Thus ringing must have greatly reduced the amount of transpiration from the ringed branch. As phosphorus must have entered the ringed branch in the transpiration current, the amount of phosphorus entering this branch must also have been greatly reduced. In spite of this the ringed branch contained more phosphorus than the normal branch (branch B).

## IV. DISCUSSION

It seems clear that whether a ringed branch will contain more or less of a phloem-mobile element than a corresponding normal branch on the same plant will depend on how seriously ringing interferes with the movement of the transpiration current. Trapping by the ring will tend to raise, and interference with the movement of the transpiration current to diminish, the amount of phloem-mobile element in the ringed branch. In the experiments of Curtis and of Clements and Engard the proportion of ringed to unringed branches would appear to have been much smaller than in our experiment, where of two branches, one was ringed. The ringed branches would appear to have had to contend with much more competition in the experiments of Curtis and of Clements and Engard than in ours. If this explanation is correct, it would appear that, depending on the proportion of ringed to normal branches, ringing may either increase or decrease the content of a phloem-mobile element. On the other hand, it will always diminish the content of a phloem-immobile element.

Our experiment would seem to be in harmony with the view that the bulk

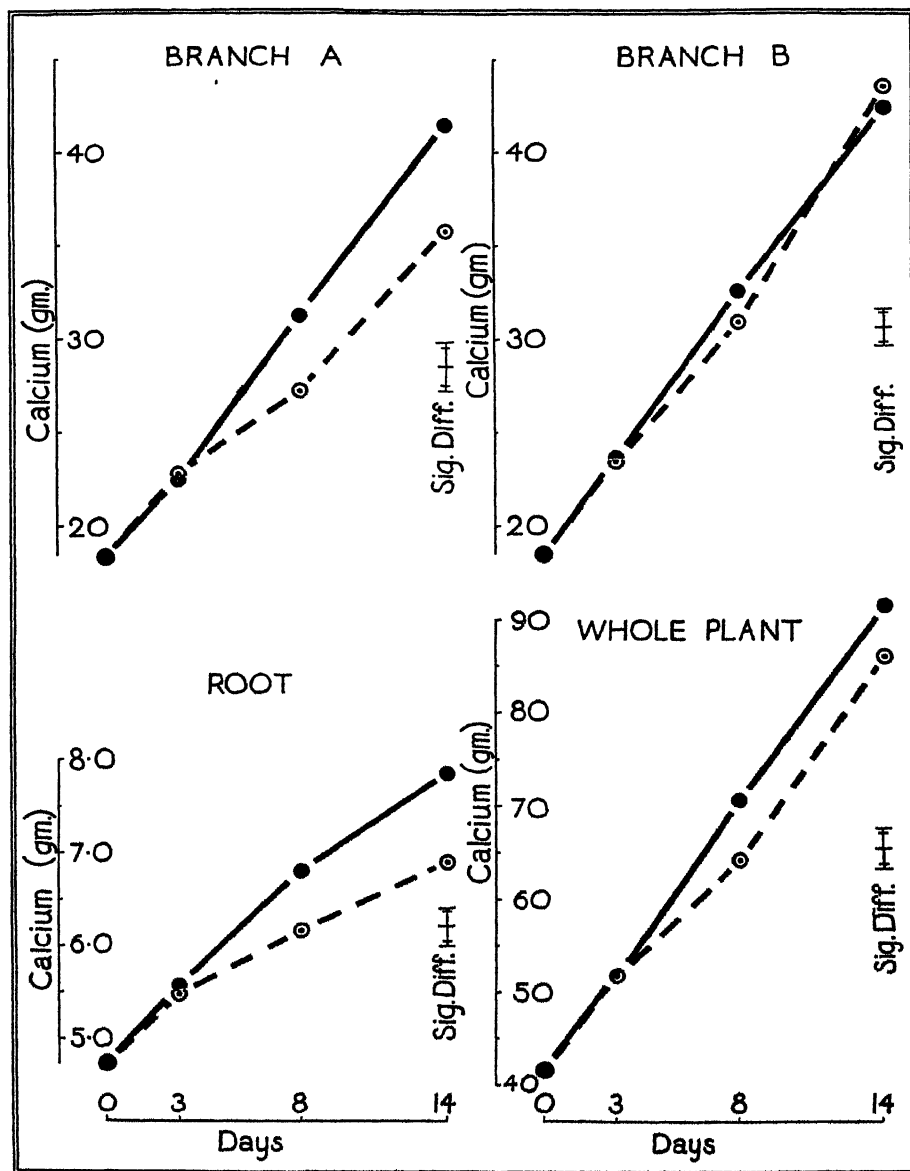


FIG. 4. Showing weights of calcium in whole plant and in regions per sample of 25 plants for the Ringed group (broken line) and for the Normal group (continuous line).

of the soil solutes normally ascend the stem via the wood and, after arrival in the leaf, may be re-exported through the phloem. Whether they can also ascend in the phloem and whether, under normal conditions, a proportion of the soil solutes actually does so is a matter we hope to consider in a subsequent paper.

#### V. SUMMARY

Plants consisting of two branches, a short main stem, and a root were employed. In one group one of the two branches was ringed. In a second group neither of the branches was ringed.

Ringing one branch caused a small reduction in the uptake of calcium and phosphorus by the whole plant.

The ringed branch contained more phosphorus and less calcium than the normal branch of the Ringed group. It also contained more phosphorus and less calcium than the branches of the Normal plant.

It was concluded that the results are in harmony with the view that the bulk of the soil solutes ascend the stem in the wood and, provided they are mobile in the phloem, are re-exported down the stem in the phloem.

It is suggested that the discrepancy between the results presented in the present paper and those of Curtis is due to the fact that the ringed branches in his experiments had to compete for water with a number of unringed branches, while in our experiment the ringed branch had only one unringed branch in competition with it.

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# The Effect of Ringing and of Transpiration on Mineral Uptake<sup>1</sup>

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## I. INTRODUCTION

THE effect of transpiration on mineral uptake has been studied for many years. Wright (1939) has referred to the earlier work, so it is only necessary for us to stress the fact that no one seems as yet to have succeeded in separating the transpiration factor from the assimilation factor. Reference should, however, be made to the work of Schmidt (1936), who investigated the effect of variation in light and humidity on the mineral uptake by the root. He concluded that there is an intimate relation between dry weight and mineral uptake by the root and that the latter was only indirectly related to water uptake. The important part played by metabolism in determining the accumulation of soil solutes by the roots has been stressed by Prevot and Steward (1936) and by Hoagland (1937).

Very recently Wright has concluded 'that an increase in the rate of transpiration is associated with a corresponding increase in the absorption of certain ions'. He tried to offset the contributory effects of assimilation upon mineral absorption, but whether or not he succeeded cannot be deduced from his data,

<sup>1</sup> Paper No. 22 from the Physiological Department of the Cotton Research Station, Trinidad.

for he failed to ascertain whether the rate of assimilation had been affected by the change in humidity to which his plants were exposed. In view of the importance of the guard-cells for both transpiration and assimilation, it seems doubtful whether the effects of the two processes on mineral uptake by the root can be separated except by isolation of the root system, *so far as phloem transport is concerned*, by the removal of a ring of bark.

*A priori* considerations suggest that the root is in a large measure dependent upon export from the foliage region for the metabolic materials on which mineral uptake depends. By removal of a ring of bark between the foliage region and the root it ought therefore to be possible to eliminate the effects of export from the foliage region. In the present paper we have tried first of all to ascertain experimentally what are the effects of ringing on mineral uptake by the root, and how soon uptake is affected after ringing. We have tried secondly to find out how transpiration affects uptake in ringed plants.

Our experiments continued for periods of only a few hours. As short-period experiments restrict the amount of material absorbed, experiments involving the uptake of an element already in the plant would be doomed to almost certain failure owing to the difficulty of measuring small increments accurately. The use of a solute not already in the plant thus becomes necessary. Bromine has accordingly been used as it is rapidly absorbed by the plant in large amounts without injury and has the further advantage that an accurate and specific method (Hibbard, 1926) is available for its determination.

## II. THE EFFECT OF RINGING ON UPTAKE (EXPERIMENT 1)

In this experiment it is shown that ringing interferes with the uptake of bromine by the root within a period of a little over two hours from the time of ringing. It is also shown that during this period the wood alone can transmit up the stem approximately as much bromine as can the wood and bark together.

### A. Procedure.

Sea Island cotton plants were grown in sand cultures receiving a complete nutrient solution from which all bromine was excluded. Two plants were grown in each container. When the plants were eight weeks old 28 containers were selected in which the two plants were closely alike. The plants were then graded to give four groups each of seven containers. In each group the taller plant in the container was marked, alternately, with black or with yellow wool, and the other plant in the container in the opposite colour. Thus each group of seven containers had seven plants tagged yellow and seven tagged black. Subsequent treatment was as follows:

Day 0, 3 p.m.

Placed in dark room. Sand leached with water and then treated with a culture solution to which potassium bromide (2 gm./litre) was added.

- „ 1, 8 a.m. One group of containers collected, the seven yellow-wooled and the seven black-wooled plants forming the A and B samples respectively of the Initial collection.
- „ 8.15 a.m. Black-tagged plants ringed at cotyledonary node and ring covered with adhesive tape.
- „ 8.30 a.m. All plants brought from dark room into the open.
- „ 10.30 a.m. Collection of three samples each of Normal (A, B, and C) and Ringed (A, B, and C) plants.

All unringed plants were ringed at the cotyledonary node immediately before collection. Collection consisted in cutting off the whole top region at the ring and then separating the two roots in each container by washing away the sand.

# **B. Results.**

(a) *Tops.* The bromine content of the whole region above the cotyledonary node, where the ring was situated in the Ringed Group, is shown in Table I. It will be seen that there was considerable movement of bromine into the Tops of the plants during the two hours' exposure to sunlight. The bromine content at 10.30 a.m. was three times as great as at 8 a.m. There was, moreover, no appreciable difference between the amounts of bromine that moved into the Tops of the two groups. It would thus appear that for some time after ringing the wood alone can transmit approximately as much bromine as the whole stem.

TABLE I

*Bromine Content of Whole Region above Ring (Tops) for Initial, Normal, and Ringed Collections. Results expressed as mg. per Sample of Seven Plants*

	Initial.	Normal.	Ringed.
A . . . . .	19.44	55.43	50.16
B . . . . .	18.76	58.32	60.00
C . . . . .	—	57.54	59.72
Mean . . . . .	19.70	57.10	56.63
Increase over Initial . . . . .		38.00	37.53

In long-period experiments such as those carried out by Curtis (1923) the efficiency of the wood and the transpiring power of the leaf may be affected, and conclusions drawn concerning the part played by the wood in the upward movement of soil solutes from the root are of course invalid.

(b) *Roots.* The weight of bromine in the region below the ring, which is referred to as the Roots, is shown in Table II.

TABLE II

*Bromine Content of Region below Ring (Roots) for Initial, Normal, and Ringed Collections. Results expressed as mg. per Sample of Seven Plants*

	Initial.	Normal.	Ringed.
A . . . . .	122.5	121.9	111.0
B . . . . .	106.1	129.0	111.1
C . . . . .		126.5	105.2
Mean . . . . .	114.3	125.8	109.1
Change relative to Initial . . . . .		+11.5	-5.2



As the Tops of the two groups showed no appreciable difference in their bromine content we may take the bromine content of the Roots as an indication of the absorptive capacities of the two groups. Reference to Table II shows that the Normal group contained greater quantities of bromine than the Ringed group. It seems clear that within a period of only *two hours* ringing has interfered with the uptake of bromine by the root. The roots are evidently very dependent on export from the Tops for their supply of materials on which mineral uptake depends. It should be added that the distance between the centre of gravity of the root system and the foliar system was approximately 18 in.

### III. TRANSPIRATION AND MINERAL ABSORPTION BY RINGED PLANTS (EXPERIMENT 2)

In this experiment the absorption of bromine by ringed plants kept in the dark was compared with the absorption by similar plants exposed to sunlight. It was found that exposure to sunlight greatly increased absorption.

#### A. *Procedure.*

The plants were grown in water cultures and were supplied with a full nutrient solution containing no bromine. The culture jars of the exposed plants were protected from the sun so that the temperatures of the exposed and darkened culture solutions did not differ by more than 1° C.

The experiment began when the plants were seven weeks old. The culture solution was then replaced by one to which potassium bromide was added. Each container held 2½ litres. There were seven samples each of eight plants. One sample served as a control to confirm the initial absence of bromine. The time table for the experiment was as follows:—

- Day 0, 10 p.m. All plants transferred to a culture solution containing potassium bromide (1 gm./litre) and placed in a dark room.
- „ 1, 5 a.m. All plants ringed at cotyledonary node and weighed with containers. Three samples placed in open and three remained in dark.
- „ 2 p.m. Plants re-weighed and collected for analysis.

#### B. *Results.*

The mean loss in weight of the plants in the open was 95.5 gm. per plant, while that of the darkened plants was only 9.9 gm. Transpiration in the open was thus many times greater than in the dark. The plants in the open showed an increase in dry weight during the day, while those in the dark decreased, the difference between the two at the time of collection being 14.8 per cent. or 3.49 gm./sample of eight plants. The weights of bromine in the Tops, Roots, and Whole Plant are shown in Table III. It will be seen that the weights were much greater in the open than in the dark groups. It thus seems clear that the

uptake of bromine is markedly affected by the rate of transpiration. It will also be seen that almost the whole of this difference was due to the Tops.

TABLE III

*Bromine Content of (Ringed) Plants under High and Low Transpiration. Results expressed as mg. Bromine per Sample of Eight Plants*

		Tops.	Roots.	Tops/Roots.	Whole plant.
Open (High Transpiration)	{ A	75.9	37.8	2.01	113.7
	{ B	83.9	49.1	1.71	133.0
	{ C	68.1	36.4	1.87	104.5
	Mean	76.0	41.1	1.86	117.1
Dark (Low Transpiration)	{ A	43.6	40.9	1.07	84.5
	{ B	47.1	40.4	1.17	87.5
	{ C	40.1	42.5	0.94	82.6
	Mean	43.6	41.3	1.06	84.9

#### IV. DISCUSSION

In past experiments on the effect of transpiration on mineral uptake by the root the effect of export of metabolites from the foliage region to the root has always obtruded itself as a disturbing factor. While there is evidence that root metabolism may influence mineral uptake, and while root metabolism must be affected by export from the leaves, there has always been doubt concerning the importance of such export in mineral uptake. In experiment 1 we have shown that the uptake of bromine by the root may, within a period of only two hours, be checked by ringing. It seems clear therefore that the supply of metabolites from the leaves must be a highly important factor in determining the rate of mineral uptake by the root.

In previous papers (1936, 1937*a*) we suggested that solute uptake by a tissue will depend on the solvent capacity of its protoplasm. Protoplasm (1937*b*, 1939) is viewed as a non-aqueous liquid with solubilities quite different from those of water. It was suggested that the solvent capacity of protoplasm is determined by its structure, and that the maintenance of this structure is dependent on a continuous supply of metabolic energy. Thus the solvent capacity of the root will be influenced by some or all of the metabolites exported from the foliage region.

In experiment 2 we eliminated the 'export' factor by comparing the uptake of bromine by ringed plants kept in the dark with that of similar plants exposed to sunlight. It was found that within a period of approximately seven hours the exposed plants had taken up much more bromine than the plants kept in the dark. It thus seems clear that in the absence of the 'export' factor transpiration affects mineral uptake. Moreover, as both assimilation and transpiration will affect mineral uptake, and as both processes are normally influenced by stomatal aperture, the transpiration effect can only be determined by ringing or possibly by cutting off the supply of carbon dioxide to the leaves.

## V. SUMMARY

1. Removal of a ring of bark between the foliage region and the root was found to depress the uptake of bromine by the root within a period of a little over two hours from the time of ringing.
2. The wood alone in the ringed plants transmitted as much bromine up the stem as the bark and wood together in the normal plants.
3. Increased transpiration caused increased uptake of bromine in ringed plants, and a greater proportion of the absorbed bromine was carried into the aerial parts of the plant under high transpiration than under low.
4. It is presumed that transpiration affects mineral uptake by altering the concentration in the absorbing region of the root and possibly also by oxygenating the root, while assimilation affects uptake by altering the solvent capacity of the root.

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# Studies in the Indian Aizoaceae

BY

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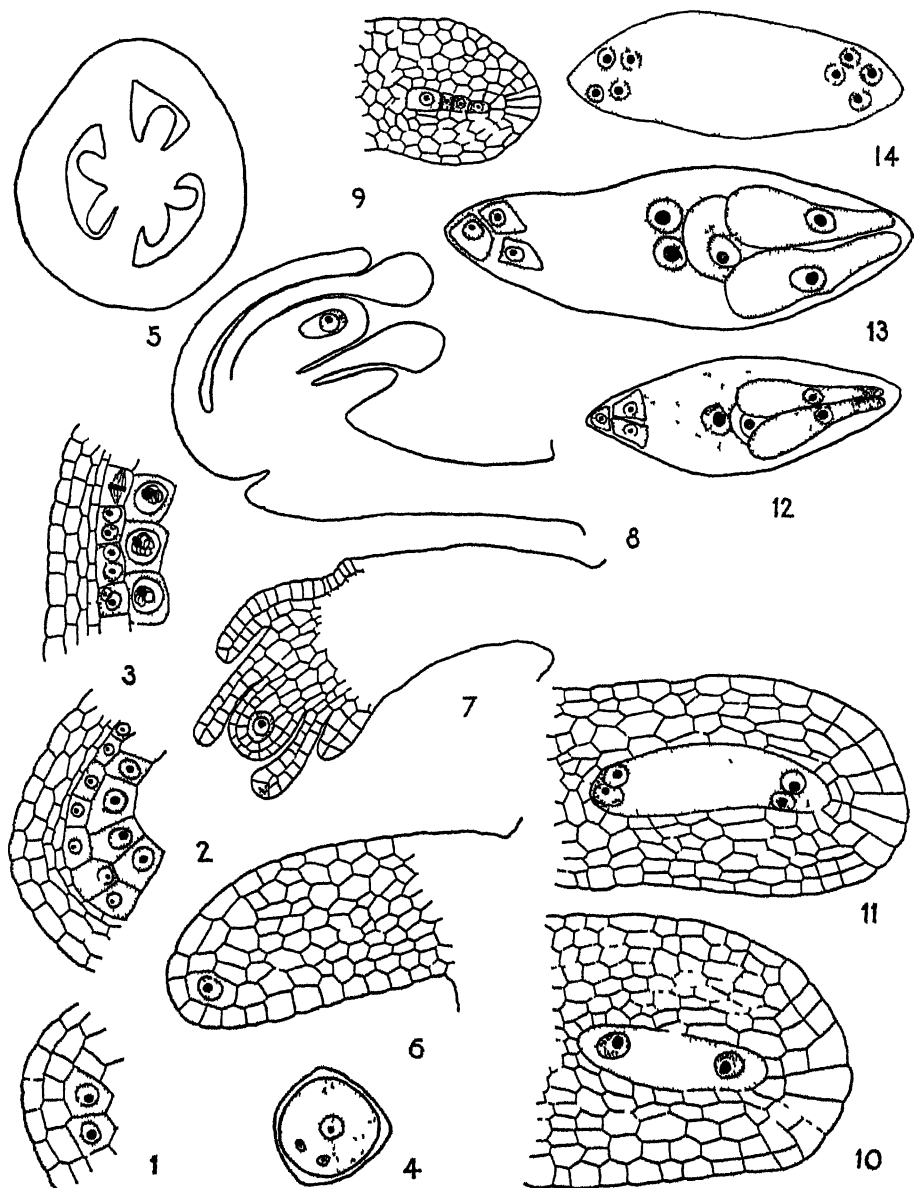
(Department of Botany, Annamalai University, India)

With sixty-four Figures in the Text

MORPHOLOGICAL work on members of the Aizoaceae is comparatively meagre. Bhargava (1934) has given a review of work on the Aizoaceae up to 1934 and has described the morphology of *Mollugo nudicaulis*, and in a later paper (Bhargava, 1935) he has given an account of the life-history of *Trianthema monogyna*, in which for the first time in the Aizoaceae he has recorded the presence of a third integument or aril. Joshi and Rao (1936) have given a summary of work on the Aizoaceae and on the allied family Phytolaccaceae and they have also described the embryology of *Gisekia pharnaceoides*, and placed these genus in the sub-family Molluginoideae. Chromosome numbers have been determined only for some species of Mesembryanthemum and a few species of Mollugo (Sugiura, 1935, 1936, 1937a) and of Trianthema (Hagerup, 1932). In the present paper chromosome numbers of eight tropical species distributed over four genera have been determined for the first time. The chromosome number of *Mollugo cerviana* has been confirmed. In addition the life-history of *Sesuvium portulacastrum* has been worked out in detail and compared with that of *Trianthema decandra*, a species hitherto not investigated. Both the genera belong to the Ficoideae group of the Aizoaceae. The morphology of members of the Ficoideae has been compared with that of the Molluginoideae.

## MATERIAL AND METHODS

All the materials for the present work were collected from plants grown in the University Botanical Gardens, Annamalaiagar, except *Sesuvium portulacastrum*, materials of which were obtained from a near-by coastal town of Porto Novo. Anthers were examined in aceto-carmin, and those which showed division stages were fixed in Karpechenko's Navashin without prefixation. For morphological work formalin acetic alcohol and corrosive sublimate fixatives were used. For older stages in the development of the embryo ovules were dissected out and then fixed. Mature seeds were soaked in glacial acetic acid for a number of days before fixing. Iron alum haematoxylin



FIGS. 1-14. *Sesuvium portulacastrum*. Fig. 1. T.S. of wall of young anther showing archesporium and two layers of wall-cells. ( $\times 750$ .) Fig. 2. Shows the five-layered anther wall. ( $\times 750$ .) Fig. 3. Binucleate tapetum. Note the mitotic division of the tapetal nucleus. ( $\times 750$ .) Fig. 4. Trinucleate pollen grain at the time of shedding. ( $\times 1,500$ .) Fig. 5. Tricarpeal ovary, with two rows of ovules on axile placenta. ( $\times 150$ .) Fig. 6. T.S. of ovule showing hypodermal archesporial cell. ( $\times 750$ .) Fig. 7. Wall-cell formation and the origin of the integuments. ( $\times 350$ .) Fig. 8. Megaspore mother cell. Note the origin of the aril. ( $\times 700$ .) Fig. 9. Linear tetrad of megaspores. (Figs. 10 and 11. 2- and 4-nucleate embryo-sacs. ( $\times 750$ .) Fig. 12. 7-nucleate embryo-sac. ( $\times 700$ .) Fig. 13. Mature embryo-sac. ( $\times 1,200$ .) Fig. 14. 8-nucleate embryo-sac. ( $\times 1,200$ .)

and Newton's iodine gentian violet were the stains employed. Sections were cut at a thickness varying between 5 and 14  $\mu$ .

*SESUVIUM PORTULACASTRUM* L.

This xerophyte is popularly known as Sea Purslane, and occurs in saline marshy tracts.

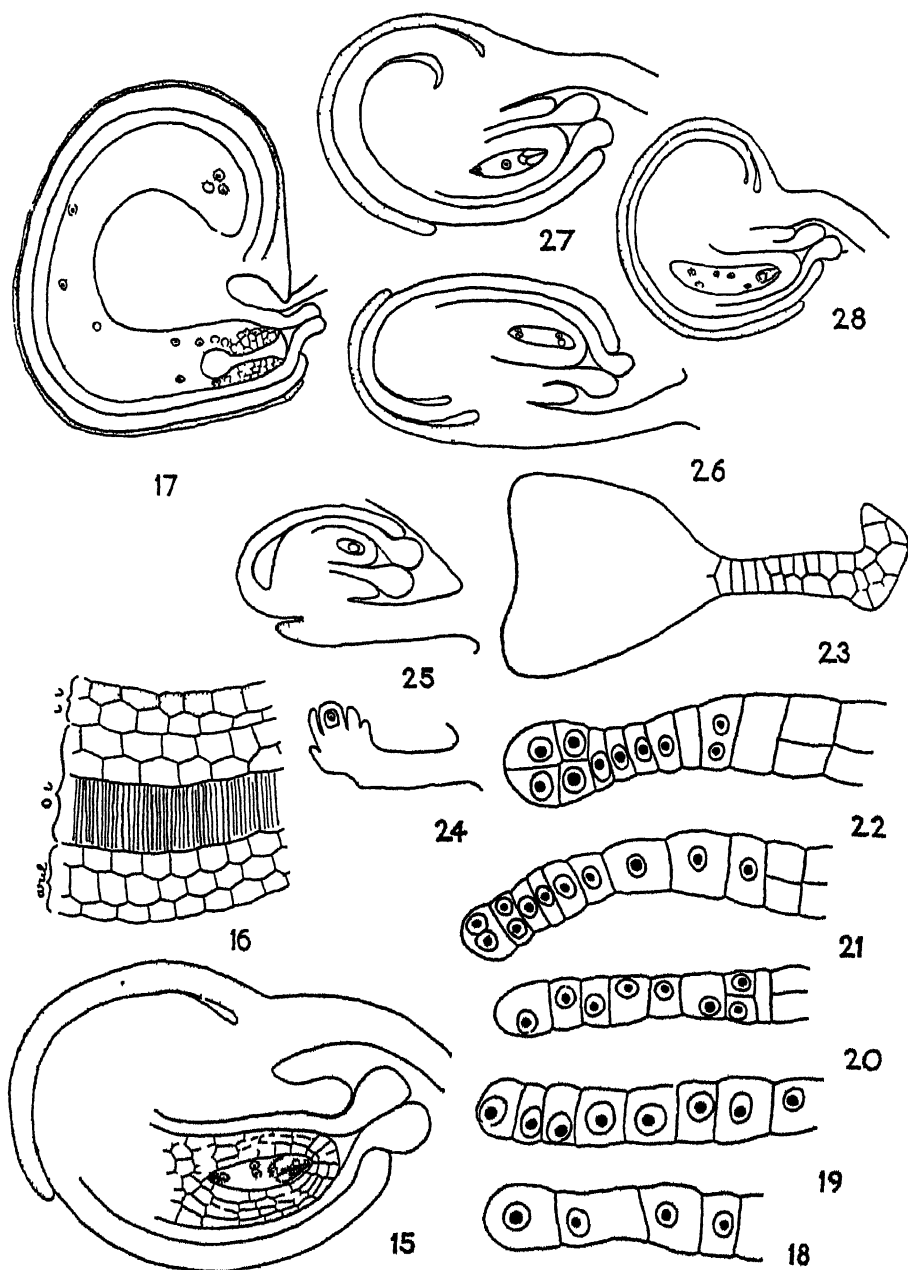
*Anther.*

Fig. 1 shows a band of two archesporial cells, with a layer of wall-cells cut off to the outside. The latter by repeated periclinal divisions gives rise to four layers, i.e. the hypodermal endothecium, two middle layers, and the innermost wall-layer, which functions as the tapetum (Fig. 2). The anther wall including the epidermis is thus five-layered. The anther wall in *Trianthema decandra* is also five-layered and a similar five-layered anther wall has been reported by Bhargava (1935) in *Trianthema monogyna*. The other Aizoaceae are reported to have only four layers of wall-cells. The single nucleus of the tapetal cells divide mitotically (Fig. 3) when the pollen mother cells are in the prophase of the heterotypic division, and the tapetal cells become binucleate. The pollen grains are polymorphous; pollen sterility is about 15 per cent. The pollen grains at the time of shedding have three nuclei (Fig. 4).

*Ovule.*

The ovary is tricarpeillary with two rows of ovules in each carpel (Fig. 5). The placentation is axile (Fig. 5). The ovule at the time of fertilization is anacampylotropous (Fig. 15). There are two integuments each of which is, to begin with, composed of two layers of cells, but during the later stages in the development of the ovules the outer integument becomes three-layered by the division of its inner layer of cells. In this respect the genus *Sesuvium* resembles *Trianthema monogyna* (Bhargava, 1935), in which alone a similar outer integument composed of three layers of cells is reported. The inner integument alone takes part in the formation of the micropyle, and at this end it is globular and composed of many cells (Fig. 15). Besides these two integuments there is a third integument or aril composed of about three layers of thin-walled cells. These three integuments are shown in Fig. 16, where the layer which became thickened and hardened in the mature seed are shaded or stippled.

The hypodermal archesporium is differentiated in the ovule very early in its development, even before the initiation of the integumental primordia (Fig. 6). The archesporial cell cuts off a parietal cell (Fig. 7). By this time the two integuments are initiated and grow rapidly and at the same time the ovule undergoes the anatropous curvature (Fig. 8). The primordium of the third integument or aril can also be seen (Fig. 8), and it grows with the growth of the ovule. The megaspore mother cell (Fig. 8) enlarges in size, before it



FIGS. 15-28. *Sesuvium portulacastrum*. Fig. 15. The ovule at the time of fertilization. Note the aril. ( $\times 220$ .) Fig. 16. Shows a section through the three integuments. ( $\times 1,500$ .) Fig. 17. Development of endosperm. Wall formation commences from the micropylar end. ( $\times 75$ .) Figs. 18-22. Various stages in the development of the embryo. ( $\times 750$ .) Fig. 23. The lobing of the cotyledons. ( $\times 355$ .) Figs. 24-28. Ovules in various stages of development, showing the development of the aril. (Figs. 24-6  $\times 150$ , Fig. 27  $\times 110$ , Fig. 28  $\times 75$ .)

divides to give rise to a linear tetrad of megaspores (Fig. 9). The chalazal megaspore is the functioning one and develops into the mature embryo-sac. The development of the 8-nucleate embryo-sac is quite normal. The various stages in the development of the embryo-sac is shown in Figs. 10-14. The nucellus immediately above the embryo-sac consists of about two to three layers of radially elongated cells, while on the sides of the embryo-sac it is composed of five to six layers of cells (Figs. 10 and 11). The 8-nucleate embryo-sac consists of two large synergids, an egg-cell, two large-sized polar nuclei, and three antipodals (Fig. 13). The synergids as well as the antipodals are ephemeral and degenerate after fertilization.

### *Endosperm.*

The endosperm nucleus divides before the division of the oospore. At first the development is free-nuclear, and the free nuclei are distributed along a peripheral layer of cytoplasm, which is rather dense towards the chalazal end (Fig. 17). Wall formation commences from the micropylar end (Fig. 17).

### *Embryo.*

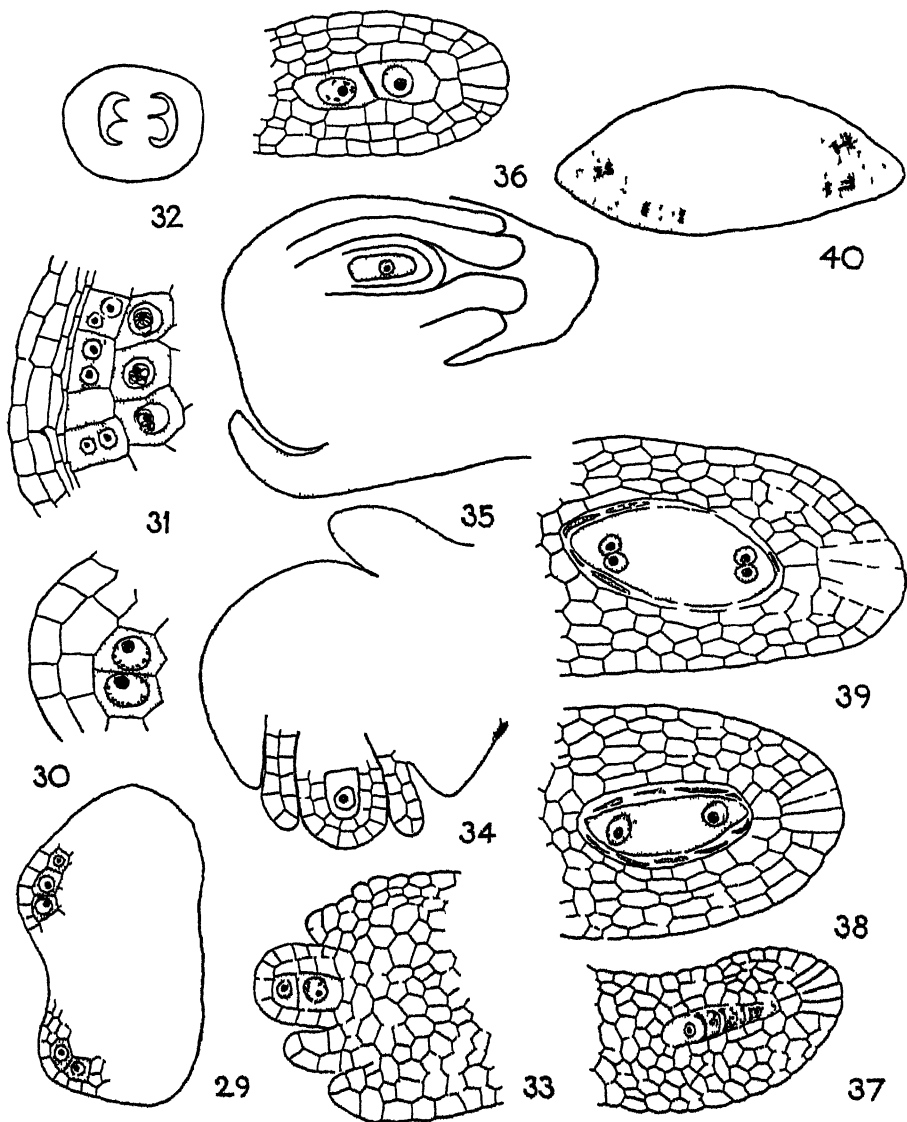
By repeated transverse divisions of the oospore a linear proembryo eight cells long is formed (Fig. 19). The two terminal cells of the pro-embryo alone take part in the formation of the embryo proper, while the third cell functions as the hypophysis. The two apical cells undergo longitudinal divisions, to form the octant (Figs. 21 and 22). Fig. 23 shows the lobing of the cotyledons. The suspensor at this stage is about twelve cells long. It remains uniseriate towards the embryonic mass, while above it is two cells broad and at the base it is multi-seriate and possesses a curved shape. In *Trianthema monogyna* (Bhargava, 1935), species of *Mesembryanthemum*, and *Tetragonia expansa* (Dahlgren, 1916) massive and several-seriate suspensors have been reported. In *Mollugo nudicaulis* (Bhargava, 1934), however, the suspensor is uniseriate.

## *TRIANTHEMA DECANDRA* L.

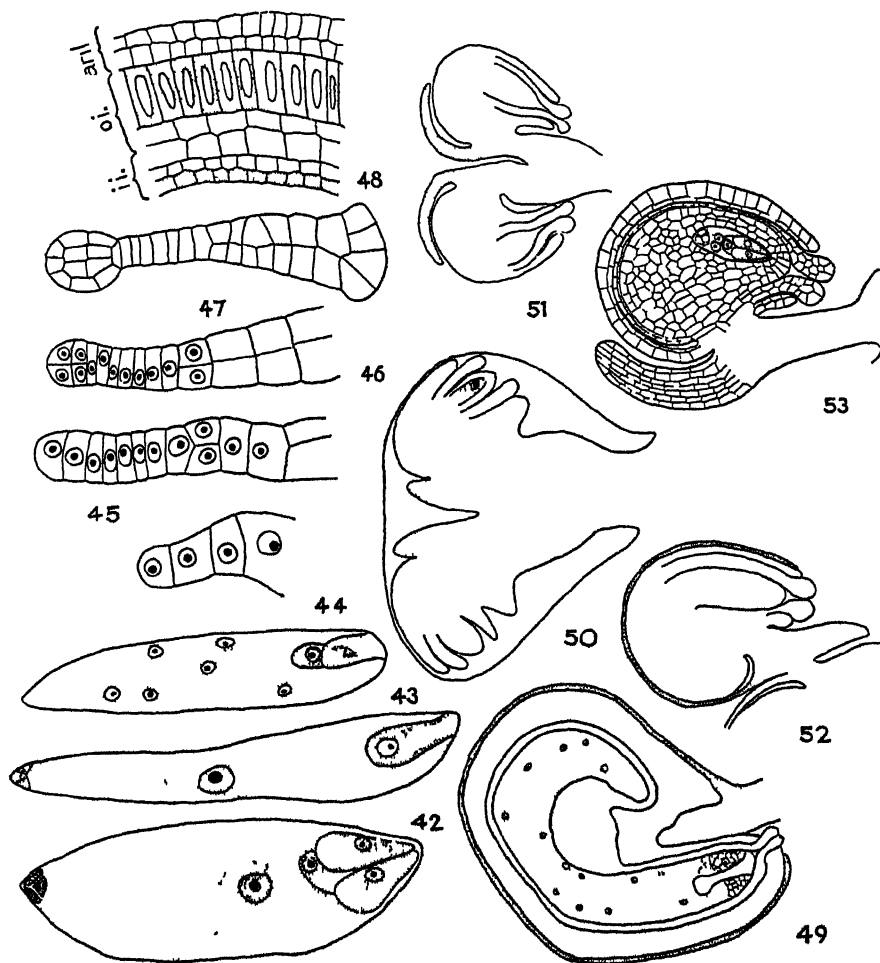
The development of the anther wall and the tapetum is similar to that of *Sesuvium*. A hypodermal band of two or three archesporial cells is present (Fig. 29), which cuts off towards the outside a layer of primary parietal cells (Fig. 30). The primary parietal cells divide repeatedly to form four layers of wall-cells. The anther wall including the epidermis is five-layered (Fig. 31). The tapetal cells are binucleate (Fig. 31). The pollen grains are trinucleate at the time of shedding.

The ovary in *Trianthema decandra* is bicarpellary (Fig. 32), with two ovules in each carpel attached to an axile placenta. The ovule at the time of fertilization is anacampylotropous. In this genus also a hypodermal archesporium, after cutting off a parietal cell (Fig. 33), functions as the megaspore mother cell (Figs. 34 and 35) and undergoes the reduction divisions to give rise to a linear tetrad of megaspores (Fig. 37), the chalazal one of which develops into





FIGS. 29-40. *Trianthema decandra*. Fig. 29. T.S. of anther showing hypodermal band of two or three archesporial cells. ( $\times 150$ .) Fig. 30. Archesporial cells with a layer of wall-cells cut off to the outside. ( $\times 1,500$ .) Fig. 31. Anther wall five cells thick. Tapetal cells binucleate. ( $\times 750$ .) Fig. 32. Bicarpellary ovary with two ovules in each carpel. ( $\times 150$ .) Fig. 33. Primary archesporial cell cutting off a wall-cell. ( $\times 750$ .) Fig. 34. Megaspore mother cell and integuments. ( $\times 750$ .) Fig. 35. Origin of aril. ( $\times 355$ .) Fig. 36. Pair of megaspores. ( $\times 750$ .) Fig. 37. Linear tetrad. ( $\times 500$ .) Figs. 38 and 39. 2- and 4-nucleate embryo-sacs. ( $\times 750$ .) Fig. 40. The four nuclei of the 4-nucleate embryo-sac dividing simultaneously. ( $\times 1,100$ .)



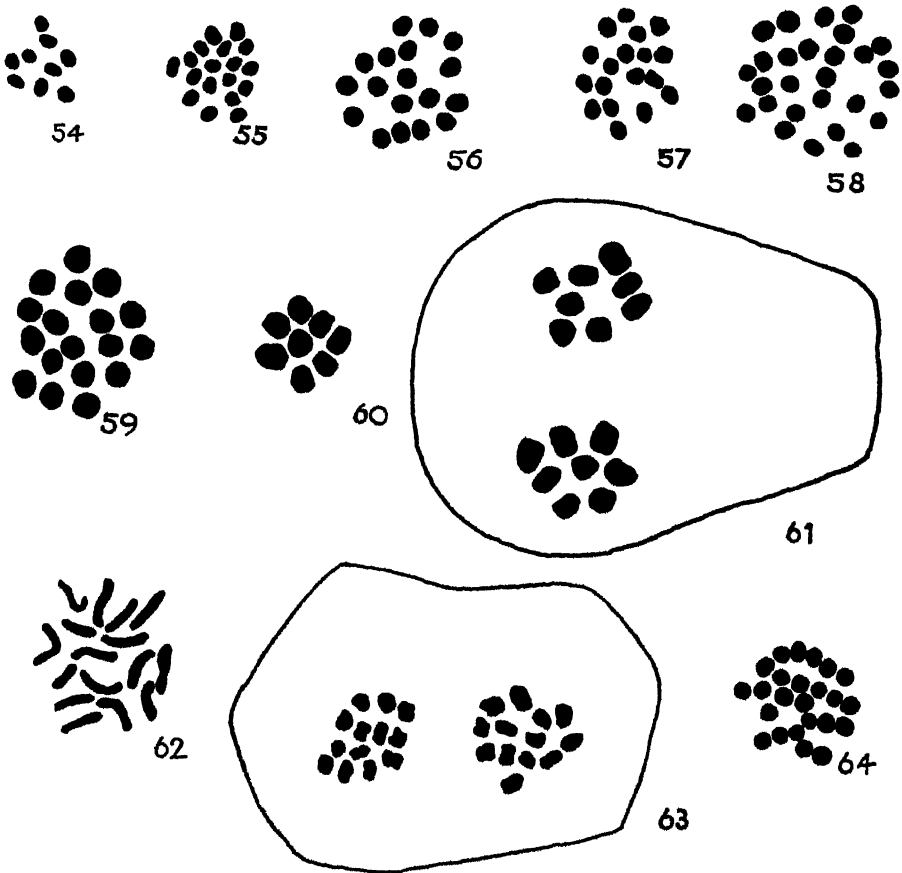
FIGS. 41-52. *Trianthema delandrea*. Fig. 41. 7-nucleate embryo-sac. ( $\times 750$ .) Fig. 42. Post-fertilization embryo-sac. ( $\times 750$ .) Fig. 43. Development of embryo and endosperm. ( $\times 355$ .) Figs. 44-7. Various stages in the development of the embryo. (Figs. 44 and 45.  $\times 750$ , Figs. 46 and 47  $\times 500$ .) Fig. 48. Structure of the three integuments. ( $\times 1,500$ .) Fig. 49. Development of endosperm. Wall formation begins at the micropylar end. ( $\times 75$ .)

FIGS. 50-2. Various stages in the development of the aril.

FIG. 53. *Mollugo oppositifolia*. Ovule at the time of fertilization. Note the radial elongation of the cells of the nucellar epidermis. Both integuments composed of two layers of cells. The aril is short and stout. ( $\times 355$ .)

the embryo-sac in the usual manner. As in *Sesuvium*, the nucellus immediately above the embryo-sac consists of two or three layers of cells, while that on the sides of the embryo-sac is about five cells thick (Figs. 38 and 39). The nucellar epidermal cells above the embryo-sac do not divide but simply stretch out radially (Figs. 38 and 39). Besides the two integuments, the outer of which becomes three-layered after fertilization, there is a third integument, the aril,

composed of two layers of thin-walled cells. The various stages in the development of the aril are shown in Figs. 50 to 52. On a comparison of these figures with those of *Sesuvium* (Figs. 24–8) it will be found that the aril in *Trianthema*



FIGS. 54–64. Fig. 54. Metaphase I in *Mollugo cerviana*,  $n = 9$ . ( $\times 3,900$ .) Fig. 55. Metaphase I in *Mollugo racemosa*,  $n = 18$ . ( $\times 2,700$ .) Fig. 56. Metaphase I in *Mollugo oppositifolia*,  $n = 18$ . ( $\times 3,900$ .) Fig. 57. Metaphase I in *Mollugo pentaphylla*,  $n = 18$ . ( $\times 3,000$ .) Fig. 58. Metaphase I in *Mollugo nudicaulis*,  $n = 27$ . ( $\times 4,500$ .) Fig. 59. Metaphase I in *Gisekia pharnaceoides*,  $n = 18$ . ( $\times 4,500$ .) Fig. 60. Metaphase I in *Trianthema decandra*,  $n = 8$ . ( $\times 3,000$ .) Fig. 61. Metaphase II in *Trianthema decandra*. ( $\times 3,900$ .) Fig. 62. Somatic metaphase in *Trianthema decandra*,  $2n = 16$ . ( $\times 3,000$ .) Fig. 63. Metaphase II in *Trianthema monogyna*,  $n = 13$ . ( $\times 3,000$ .) Fig. 64. Metaphase I in *Sesuvium portulacastrum*,  $n = 24$ . ( $\times 2,225$ .)

develops more rapidly than in *Sesuvium*. It has grown fully, and completely surrounds the outer integument (Fig. 52) by the time the embryo-sac is mature, while in *Sesuvium* this happens only after fertilization (Fig. 28). Fig. 48 shows a section of the three integuments; the layers which became hard in the mature seed are indicated by stippling.

The development of the endosperm (Figs. 43 and 49) and the embryo (Figs. 43-7) is similar to that in *Sesuvium*. The suspensor in *Trianthema decandra* is not so massive and multiseriate as that figured for *Trianthema monogyna* (Bhargava, 1935).

# MOLLUGO OPPOSITIFOLIA L.

The development of the ovule in *Mollugo oppositifolia* is somewhat different from that described for *M. nudicaulis* (Bhargava, 1934). In *M. nudicaulis*, as also in *Gisekia pharnaceoides*, the nucellus above and at the sides of the embryo-sac is about four to five cells thick because of the division of the nucellar epidermal cells as well as the primary parietal cells. In *M. oppositifolia* there is no massive nucellus, and the radial stretching of the nucellar cap, characteristic of the Ficoideae group, is exhibited. In the other species of *Mollugo* so far investigated the third integument is absent; it is also absent in *Gisekia*. In *M. oppositifolia*, however, it is found that the aril is present though it does not completely envelop the ovule (Fig. 53) and is thicker and stouter, being composed of about seven layers of cells (Fig. 53). It is rather curious that this particular species of *Mollugo* should differ from the rest of the Molluginoideae in these two important matters, which as will be discussed later, may be regarded as the morphological basis for the differentiation of the two sub-families.

## Chromosome Counts.

The following chromosome numbers have been determined (Figs. 54 to 64):

Molluginoideae	<i>n</i>	Ficoideae	<i>n</i>	<i>2n</i>
<i>Mollugo cerviana</i> ser	9	<i>Trianthema decandra</i> L.	8	16
„ <i>oppositifolia</i> L.	18			
„ <i>racemosa</i> Lamk.	18	<i>Trianthema monogyna</i> L.	13	—
„ <i>pentaphylla</i> L.	18			
„ <i>nudicaulis</i> Lamk.	27	<i>Sesuvium portulacastrum</i> L.	24	—
<i>Gisekia pharnaceoides</i> L.	18			

## DISCUSSION

The family Aizoaceae is subdivided by taxonomists into the Molluginoideae and the Ficoideae according to the character of the perianth and the relative position of the ovary. The Molluginoideae has almost free perianth segments while in the Ficoideae they are united to form a tube. As there are available some morphological and cytological data, it is of interest to determine if these throw any light upon this division of the family.

The members of the Molluginoideae seem to be characterized by the following: (a) the presence of two integuments, each composed of two layers of cells; (b) the absence of the aril or third integument; (c) the nucellus above the embryo-sac is massive; (d) the anther wall is four cells thick.

The Ficoideae, on the other hand, exhibit: (a) two integuments, of which the inner integument is composed of two layers of cells, and the outer of three

layers of cells; (b) an aril enveloping the entire ovule is present; (c) the nucellar epidermal cells elongate radially and are not massive above the embryo-sac; (d) the anther wall is five cells thick.

There appears also to be a difference in the basic chromosome numbers of the two families. The correlation between chromosome numbers and systematic position has been established by a number of authors; see, for example, Eigsti (1936) for *Reseda*, Winge (1917) for certain *Compositae*, Sugiura (1939) for the *Plumbaginaceae*. The species of *Mollugo* and *Gisekia* of the *Molluginioideae* belong to the 9 series, while the species of *Trianthema* and *Sesuvium* of the *Ficoideae*, with the exceptions mentioned below, belong to the 8 series. The chromosome numbers already determined for the other members of the *Aizoaceae* by previous authors are also found to belong to one or other of these series. Since in the family *Aizoaceae* the chromosome numbers present two straightforward polyploid series, we may subdivide the *Aizoaceae* into two sub-families, the *Molluginioideae* containing the genera of *Mollugo* and *Gisekia* and the *Ficoideae* containing the genera of *Trianthema* and *Sesuvium*. This conclusion coincides with that already arrived at on the basis of morphology. From this it may be concluded that the division of the family *Aizoaceae* by Hutchinson (1926) into the *Molluginioideae* and the *Ficoideae* is justified. There are, however, a few variations from these two polyploid series. For example, *T. monogyna* has 13 as the haploid number, though the chromosome number of many other species of the genus conforms to the 8 series. Similarly *M. verticillata* has 32 as the haploid number, though many other species of the genus conform to the 9 series. Other instances of such irregular deviations are well known and have been termed dysploids (Jeffrey, 1925).

The case of *M. oppositifolia* is interesting in (1) the presence of an aril, (2) the radial elongation of nucellar epidermal cells which is a character of the *Ficoideae*, but otherwise it agrees with the other members of the *Molluginioideae*. This species may be looked upon as a connecting link between the two sub-families rather than as an exception to a general rule.

The position of the genus *Gisekia* calls for some mention. It differs from the rest of the *Aizoaceae* in possessing an apocarpous gynaecium. Modern taxonomists like Hutchinson (1926) placed it in the *Molluginioideae*, though older systematists like Baillon (1875) and Engler and Prantl (1889) brought it under the *Phytolaccaceae*. In its basic chromosome number of 9 and in anther and ovule morphology it agrees with the *Molluginioideae* in which it should therefore be placed.

The genus *Mesembryanthemum* is also interesting, from this point of view. The basic chromosome number of this genus is 9 as in the *Molluginioideae*. In the morphology of the ovule it shows some agreement with the *Ficoideae*. Thus Joshi and Rao (1936, p. 89) say that 'the cells of the nucellar epidermis just above the embryo sac do not divide but stretch out radially. . . . The embryological studies therefore support the views of Hutchinson (1926) who has placed *Ficoidaceae* (including *Mesembryanthemum*, *Trianthema* and *Tetra-*

gonia) as a separate family from the Molluginoideae.' But it is also known (Bhargava, 1934, p. 271) that *Mesembryanthemum* has no third integument, which is now found to be an important feature of the Ficoideae. It seems, therefore, reasonable not to attach too much importance to agreement in this one morphological detail, since in another such detail and in its chromosome number it does not agree with the Ficoideae. Joshi and Rao (1939, p. 39) also say 'the genus *Gisekia* in these respects agrees with *Mollugo* and *Mesembryanthemum* and should therefore be placed in the Molluginaceae'. In our view it appears reasonable to remove *Mesembryanthemum* from the Ficoideae; and if on account of its inferior ovary it is not to be placed in the Molluginoideae it can well be shifted to the Phytolaccaceae, where its chromosome number fits. A few genera in the Phytolaccaceae, like the Mexican genus *Agdestis*, possesses an inferior ovary which makes *Mesembryanthemum* not quite out of place in the Phytolaccaceae.

## SUMMARY

1. Chromosome numbers of nine species of the Aizoaceae have been determined.
2. The development of the microsporangium, the ovule, and the embryo is described in detail in *Sesuvium portulacastrum*. That of *Trianthema decandra* is found to be very similar.
3. These genera show chromosome numbers and morphological details which characterize the sub-family Ficoideae and distinguish it from the Molluginoideae.
4. *Mollugo oppositifolia*, which exhibits characters of both the sub-families, is interpreted as a connecting form between the two.
5. The systematic position of the genera *Gisekia* and *Mesembryanthemum* is discussed.

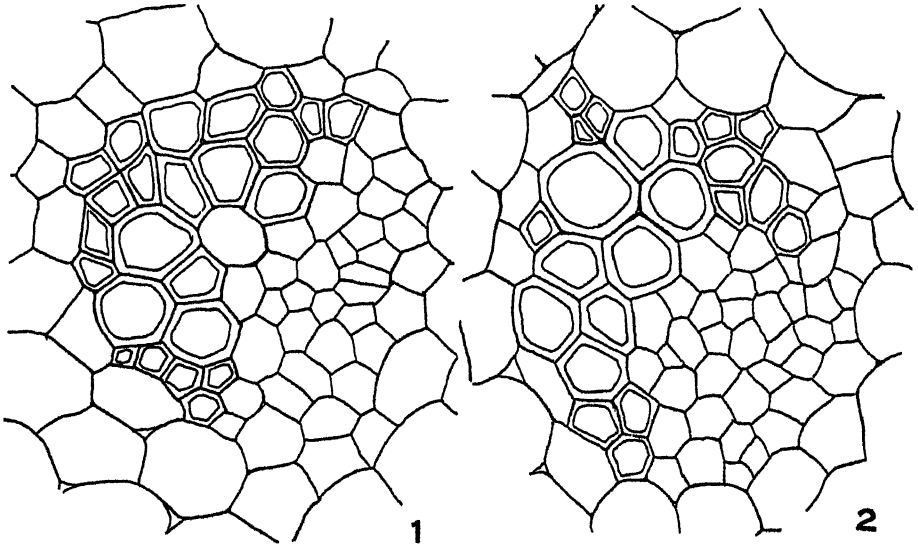
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## NOTES

**A NOTE ON THE ANATOMY OF THE ROOTS OF OPHIOGLOSSUM.**—The roots in all species of *Eu-Ophioglossum* except *O. bergianum* are monarch (Campbell, D. H., *The Eusporangiateae*, Washington, 1911; Bower, F. O., *The Ferns*, vol. ii, Cambridge, 1926). In material of *O. vulgatum* collected from the Punjab they are also found to be so, but the xylem strand shows some peculiarities



FIGS. 1 and 2. *Ophioglossum vulgatum*. Transverse sections of two roots showing the structure of the stele. ( $\times 300$ .)

which deserve mention. It has in transverse section a semi-lunar form and the smaller tracheides are found not in one but in two (Fig. 1) or three (Fig. 2) groups. The arrangement of the elements is such that it appears as if two or three radial bundles of xylem had fused together. In Fig. 1 the xylem appears diarch, in Fig. 2 triarch. Although *O. vulgatum* has a world-wide distribution and has been extensively studied in the past, I find no mention of this fact in the previous literature; perhaps the peculiarity is confined to Indian material. In any case it shows clearly that the prevailing monarch structure of the roots of *Ophioglossum* has been derived from a diarch or triarch condition, such as is found in *Helminthostachys* and *Botrychium*, by the suppression of one or more phloem strands and the fusion of the several radial xylem bundles on one side of the stele.

A corollary from the above conclusion relates to the branching of the roots in the *Ophioglossaceae*, for this shows marked correlation with the stellar structure. The diarch and triarch roots show monopodial branching, while the monarch roots branch dichotomously. It is generally held that the dichotomous method of



branching is primitive and the monopodial type has been derived from this through a stage like unequal dichotomy. The present observations, however, lead to the conclusion that in the Ophioglossaceae the dichotomous branching of the roots has been derived from the monopodial type. It is not a primitive condition but the result of reduction.

Both these conclusions concerning the root morphology of the Ophioglossaceae, the origin of monarch from polyarch structure and of dichotomous from monopodial branching, agree with the now generally accepted view of Bower, based primarily on the morphology of the stem, leaf, and spike, that 'within the family Botrychium and Helminthostachys appear to be relatively primitive genera and Ophioglossum more highly specialized'.

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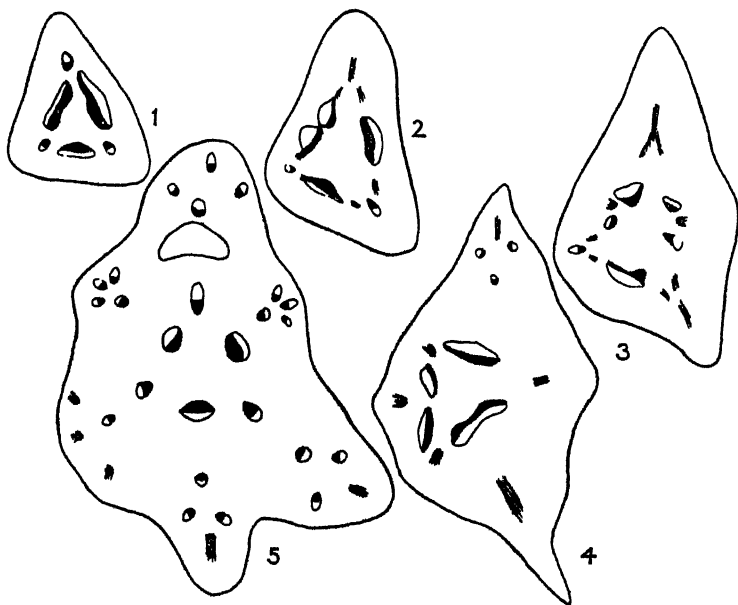
**THE CONSERVATE CHARACTER OF THE VASCULAR SYSTEM: COMPARATIVE ANATOMY OF NORMAL AND PENTAPHYLLOUS BICARPELLARY FLOWERS OF GAGEA FASCICULARIS.**—It is generally believed by morphologists that the vascular system of an organ is more conservative than the external form. As Bower ('The Filicales', vol. i, Cambridge, 1923) says, 'Anatomical characters are apt tardily to follow evolutionary progress and thereafter to persist as vestigia.' Arber ('New Phytologist', xxxii, 1933), however, takes exception to this point of view and rejects the principle of conservatism of vascular bundles. To the question, 'Are we to consider it proven that the vascular bundles are more conservative than the external form, so that vestigial organs may be represented by their bundles, when all external trace of these organs has disappeared?', her answer is that 'we have no alternative but to discard the doctrine of the conservatism of the vascular bundles'. She cites a number of examples from her extensive work on floral morphology to show the existence of organs whose vascular supply has disappeared prior to the loss of the organs themselves and says, 'there seems to be no escape from the conclusion that there is a complete absence of positive evidence for the vestigial survival of vascular tissue after the organ which it supplied has ceased to exist'. Saunders ('New Phytologist', xxxiii, 1934), on the other hand, has criticized Arber's work and upheld the general view. In a recent review, Wilson ('Bot. Rev.', v, 1939) admits that in some cases what appear to be rudimentary organs have persisted with no corresponding vascular supply; still he says, 'it may be stated with confidence that, on the whole, vascular bundles are more conservative than external forms; that in general the vascular supply to an organ persists while the structure is in the process of disappearing'. In reply to a short criticism by the writer (Joshi, 'Nature', cxxxii, 1933), Arber pointed out the need for further investigation of the subject.

During a visit to Kashmir in 1937, the writer collected some floral material of *Gagea fascicularis* Salisb. (*G. lutea* Ker-Gawl.) from Khilanmarg. Most of the flowers in this material were found to have a normal liliaceous structure. They possessed a perianth of two trimerous whorls, two trimerous whorls of stamens, and a tricarPELLARY syncarpous gynaecium consisting of a trilocular superior ovary, one style and stigma. Among these normal flowers, however, one flower was found with only five quincuncial perianth leaves, five stamens, and a bicarPELLARY gynaecium. The variation was obviously the result of loss of one perianth leaf,

one stamen, and one carpel. It appeared possible that the study of the effect of such a loss on the vascular construction of the flower may throw some light on the doctrine of the conservatism of vascular bundles, particularly with regard to its application to floral morphology. For this reason the present investigation dealing with the comparative anatomy of the normal and variant flowers was undertaken.

*Anatomy of the normal flowers.*

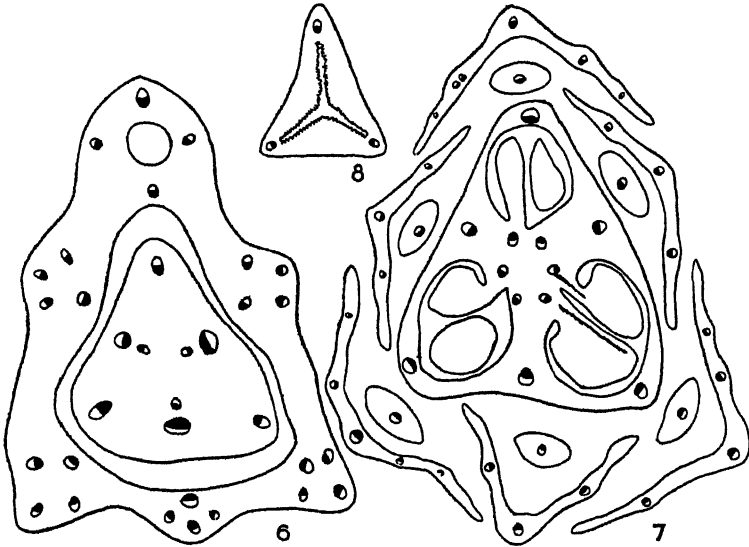
The anatomy of the normal flowers is illustrated in Figs. 1-8, which represent a series of transverse sections through a normal flower from the pedicel upwards.



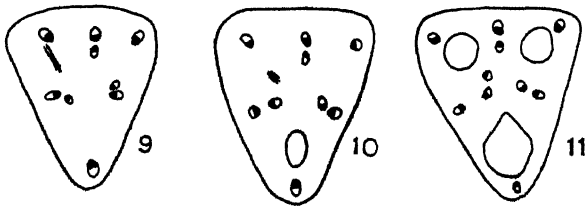
FIGS. 1-5. ( $\times 30$ .)

The pedicel has a triangular outline in cross section and shows typically six vascular bundles (Fig. 1). Three of these are small and are situated opposite to the angles, while the three bundles alternating with these are of a comparatively much larger size. As we approach the thalamus, the stele broadens out and the smaller bundles begin to pass outwards. They are further joined by two traces from the sides of the larger bundles (Figs. 2 and 3). These composite bundles form the vascular supply of the outer whorl of perianth leaves and stamens. At the level the small bundles and the lateral traces from the larger bundles begin to unite, three traces are given out from the middle of the larger bundles (Figs. 3 and 4). These form the vascular supply of the inner whorl of perianth leaves and stamens. Higher up each of these six perianth-stamen traces breaks up into four bundles (Figs. 4 and 5). Three of these are situated towards the outside and supply a perianth leaf, one forming the midrib bundle and two forming the lateral veins. The fourth one is situated towards the inside of the midrib bundle of the perianth leaf and supplies a stamen. The stamens are fused for some distance with the perianth leaves to form a short tube surrounding the base of the ovary (Fig. 6).

After the perianth and staminal vascular supply has been given off, the vascular tissue remaining in the centre of the thalamus again organizes into six bundles (Fig. 5). Three of these bundles which are on the same radii as the midrib bundles of the outer whorl of perianth leaves are smaller in size. Alternating with them



FIGS. 6-8. ( $\times 30$ .)

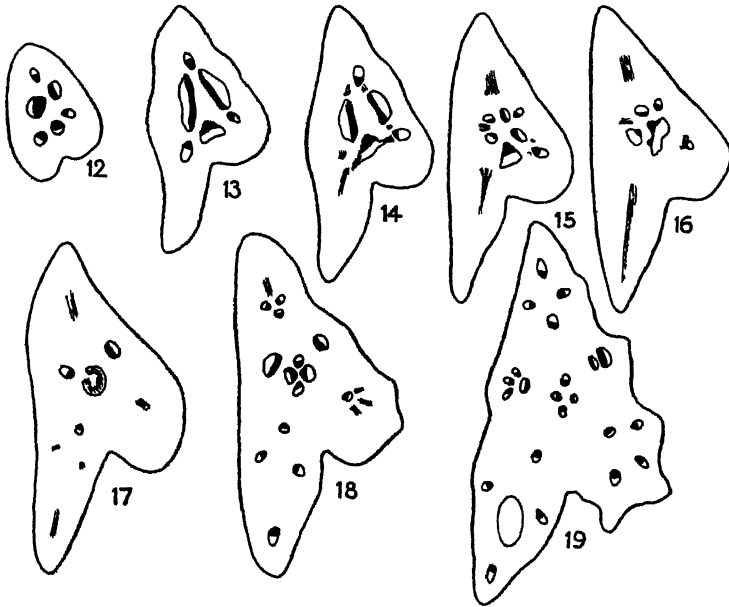


FIGS. 9-11. ( $\times 30$ .)

are three larger bundles. The latter give off towards their inside another branch (Fig. 6), which is inversely orientated. The ovary at its base thus shows nine bundles arranged in two rings. The outer ring has six normally orientated bundles. The smaller ones of these form the dorsal traces of the carpels and the alternating larger bundles are the median laterals. The inner ring has three inversely orientated bundles. These are the fused ventrals of adjacent carpels and by their division higher up form the ventral traces of the carpels (Fig. 7), which supply the ovules. At the top of the ovary, the ventral and the median lateral traces of the carpels come to an end. The style has triangular outline in transverse section and shows three bundles (continuations of the dorsal bundles of the carpels) in the three angles (Fig. 8). The styler canal is tri-radiate, of the hollow type, and lined by the glandular transmitting tissue.

Figs. 9-11 represent a series of three sections through the base of the gynaecium

of another flower with normal parts. It shows the origin of the ventral traces on two sides (the upper and the right in the figure) in the normal manner. They arise simply as branches from the median laterals, become inversely orientated by turning round as they move inwards and divide to form the ventrals of the adjacent



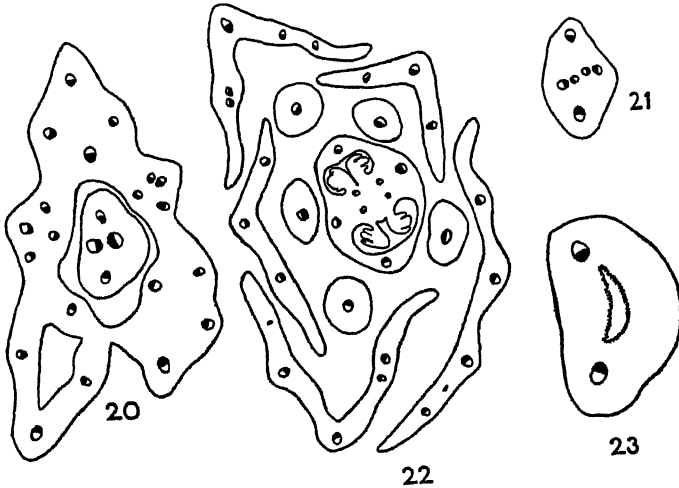
FIGS. 12-19. ( $\times 30$ .)

carpels. On the left-hand side, however, one of the ventrals is seen to arise from the median lateral bundle of that side, while the second one is given off from the dorsal bundle of the carpel. This origin of a ventral trace from the dorsal trace and not from the neighbouring median lateral is very significant and shows the plasticity of the vascular tissue.

#### *Anatomy of the abnormal flower.*

The anatomy of the flower with five perianth-leaves, five stamens, and bicarpellary gynaeceum is illustrated by Figs. 12-19 and 20-23. Figs. 12 and 13 show the structure of the pedicel. It differs from that of normal flowers in the development of a conspicuous groove on one side. The groove starts from the very base of the pedicel and continues right up to the separation of the perianth leaves. This can be easily seen by following the series Figs. 12-20. The development of the groove is obviously the result of loss of one of the perianth leaves and stamens from this side of the flower. The vascular structure of the pedicel, however, is quite similar to that of normal flowers, and traces for the outer whorl of perianth leaves and stamens are also given off in the same manner (Figs. 14-19). After the departure of these, three large bundles are left in the centre. One of these is just to the inside of the groove on the pedicel. From the middle of the other two bundles traces come out for the two inner perianth leaves and stamens in the same manner

as in normal flowers (Fig. 15), but there is absolutely no sign of any vascular trace from the groove-opposed bundle which would have supplied the missing perianth leaf and stamen. The vascular bundles of the thalamus after the departure of the perianth and stamen traces come close together and unite to form roughly a



FIGS. 20-23. (Figs. 20-22  $\times 30$ ; Fig. 23  $\times 90$ .)

ring of vascular tissue (Figs. 16 and 17). This breaks higher up into four bundles (Figs. 18-20), two large alternating with two small ones, which organize into the dorsal, median lateral, and ventral bundles of the two carpels in the same manner as in normal flowers (Figs. 21 and 22). The smaller bundles continue as the dorsal bundles. The larger ones give off ventrals towards the inside and then form the median laterals. The only notable point is that the dorsal bundles of the two carpels are not situated exactly opposite to each other as they should be in a normal bicarpellary syncarpous gynaeceum. One side of the ovary thus appears to be slightly better developed than the other (Fig. 22). In the style as in the normal flowers the ventral and the median lateral traces fade away, and only the dorsal bundles continue, but their arrangement is very lop-sided. Ordinarily in a bicarpellary syncarpous gynaeceum with a single style, the style appears in transverse section either circular or elliptical and shows two bundles facing each other in a perfectly symmetrical fashion. In the present case the style appears in transverse section nearly triangular (Fig. 23) and approaches the style of normal flowers of *Gagea fascicularis*. Further the two vascular bundles occupy two angles of the triangle, so that one side is far more developed than the other.

#### Discussion.

In recent years there has been a great revival of interest in floral anatomy, particularly due to the work of Saunders and Arber in England, Eames in America, and Troll in Germany. This work has often led to much controversy with regard to the morphology of the various floral parts, especially the gynaeceum. As a good deal of such controversy ultimately depends on how much importance one

attaches to the vascular system in the solution of morphological problems, a proper evaluation of the doctrine of conservatism of vascular bundles should be of great service to future investigations in this line. An attempt, therefore, will be made in the present and subsequent investigations to study the effect of the loss or increase in the number of parts of the flower on its vascular system.

Comparison of Figs. 2 and 3 clearly shows that the abnormal flower of *Gagea fascicularis* with 5 perianth leaves, 5 stamens, and 2 carpels has been derived from the normal flowers by the loss of one of the inner perianth leaves, one stamen and one carpel. The study of the vascular system of this flower, although it is not separated even by one generation from the normal flowers, shows the total absence of any rudimentary vascular traces of the lost parts. It is, therefore, clear that in this case no bundles persist after the organ which they supplied has ceased to exist.

The vascular plan of the flower as a whole, however, does not change so rapidly, and a great deal of resemblance is found between the vascular plans of the abnormal and normal flowers. Thus although the perianth leaves in the abnormal flower are arranged in a quincuncial manner and are nearly equally spaced around the periphery of the thalamus, their vascular traces arise in the same manner as in normal flowers, i.e. in two whorls. The two inner alternate with the outer; the only difference is the absence of one of the inner perianth-stamen traces. In the ovary it is found that the dorsal bundles of the two carpels are not exactly opposite one another and lack the symmetry seen in a normal bicarpellary gynaecium. This is particularly marked in the style. Here the two bundles, which are continuations of the dorsal bundles of the carpels, are so situated as to occupy the angles of a triangle. They have the same position as in a normal tricarpellary gynaecium—only one of the angles of the triangle is not occupied by any bundle—and indicate by their asymmetric disposition the loss of one of the carpels.

Externally the loss of one of the perianth leaves and stamens is shown by the development of a groove along the whole length of the pedicel, even though its vascular system is quite similar to that of the normal flowers. Such a groove is not seen in normal pentaphyllous flowers of other plants. Its presence, therefore, indicates recent loss of some parts. Incidentally, this fact lends support to the 'leaf-skin Theory' of Saunders (1922), according to which the outer cortical portion of the stem is formed by downward prolongation of the leaf-bases.

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**A PRELIMINARY ACCOUNT OF THE LIFE-HISTORY OF PORPHYRA UMBILICALIS (L.) AG.**—During an investigation into the phases of colonization of newly constructed concrete surfaces in the littoral zone at Mumbles Head, Swansea, it was found that *Porphyra umbilicalis* was a dominant constituent of the algal flora during both pioneer and subsequent phases. Advantage was taken of the opportunity thus provided to study its development and life-history. As soon as the ecological work is completed a full account of these observations will be published; the following is a brief summary of the life-history of this alga as it is found at Mumbles Head.

1. Although a *Porphyra* community is to be found throughout the year, the life of an individual plant rarely exceeds eight months.

2. *P. umbilicalis* exists in three different forms—the broad umbilicalis type, the narrow linearis type (= *P. linearis* Grev.), and a microscopic branched filamentous type. All three forms may produce reproductive bodies.

3. It is convenient to begin with *P. umbilicalis f. linearis*. This produces carpospores which germinate to produce short sparingly branched filaments which live about four weeks. One or more cells in these filaments liberate non-motile spores. There is evidence that at least two and probably three successive generations of these short-lived plantlets occur on the concrete surfaces.

4. Spores from these plantlets [which may be compared to the plethysmothalli of Sauvageau (Compt. Rend. Acad. Sci., clxxxv, 430, 1927)] germinate to produce an umbilicate form (type B).

5. Type B becomes fertile and produces crops of carpospores which on germination give rise to microscopic plantlets as in 3.

6. These plantlets liberate spores which germinate to produce another crop of umbilicate plants (type B).

7. Later on type B produces gonidia which, when they germinate, give rise to *f. linearis*. This completes the life-cycle, but there is also another and concurrent cycle of events taking place which may be summarized thus:

8. *f. linearis* gradually widens and eventually changes into an umbilicate form (type A), which becomes fertile, eventually producing carpospores.

9. These carpospores produce microscopic plantlets, spores from which germinate into the type B umbilicate form.

The sequence of events over a single year is as follows:

I. *August–November*: gonidia germinate to produce *f. linearis*.

*September–February*: *f. linearis* dominant but mixed with *P. umbilicalis* type B.

*December–February*: *f. linearis* produces carpospores.

*February–April*: *f. linearis* gradually changes into *P. umbilicalis* type A.

*April–July*: type A produces carpospores.

*May–August*: type A begins to decay and finally to disappear.

II. *December–February*: carpospores from *f. linearis* germinate into plethysmothalli.

*April–July*: carpospores from type A germinate to produce plethysmothalli.

*January–August*: spores released from plethysmothalli (of whatever origin) produce new crops of type B.

*March–August*: type B produces carpospores which give rise to a further crop of plethysmothalli.

*August–November*: type B produces gonidia (I have no evidence of gonidia being produced on type A).

*November–February*: type B, having produced gonidia shows signs of decay and most of the plants have disappeared by February.

Types A and B of *Porphyra umbilicalis* differ (a) in their origin, the former developing as a result of a gradual modification of *f. linearis*, the latter developing from germinating spores; (b) in the period during which they predominate as constituents of the algal flora. In external form and in details of their anatomy

(cell dimensions, position and size of carpogonia, &c.) they appear to be practically identical and have been fully described as *P. umbilicalis* by various writers (H. Kylin, 'Arkiv. f. Botanik', xvii. 1, 1922; V. M. Grubb, 'Rev. Algol.', i., 223, 1924; P. Dangeard, 'Le Botaniste', xviii. 183, 1927, 'Trav. Crypt. L. Mangin', 85, 1931) in recent years. The narrow *f. linearis* appears to be purely a winter form. The microscopic plantlets, details of the form and development of which will be given in a later full account are usually 4-14 cells in length, with two or three short branches. Cells measure  $16-20\ \mu \times 4-5\ \mu$ , and spores liberated from them are about  $11-14\ \mu$  diam. compared with a carpospore diameter of  $18-20\ \mu$ .

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# The Salt Relations of Plant Tissues

## II. The Absorption of Manganese Salts by Storage Tissue

BY

WALTER STILES

AND

A. D. SKELDING

With six Figures in the Text

### INTRODUCTION

THIS paper is the second instalment of our general survey of the course of absorption of inorganic salts by storage tissues and deals with the absorption of manganese salts by carrot root tissue. As far as we are aware there are no previous observations on this question; indeed, the only quantitative data of the absorption of manganese salts by plant tissue of any kind appear to be those of Laine (1934) on the absorption of manganese chloride by detached roots of *Phaseolus multiflorus* and the very recent ones of Collander (1939), published while the present work was in progress, on the absorption of the same salt by cells of *Chara* and *Tolypellopsis*. As, however, manganese belongs to the group of micro-nutrient elements, its importance in plant physiology, agriculture, and horticulture is now unquestioned, while there are also indications that it may also be a factor in determining plant distribution.

### MATERIAL AND METHODS

As in the examination of the absorption of potassium salts, described in the first paper of this series, carrot tissue was employed in the form of discs 2 cm. in diameter and 0.1 cm. in thickness and the discs were prepared in just the same way.

Three manganese salts, the chloride, sulphate, and nitrate, were used in concentrations ranging between 0.02 M and 0.0002 M. The first experiments were carried out in the same way as those with potassium salts, that is, 60 discs were placed in 200 ml. of solution contained in bottles of 600 ml. capacity. Samples of the solution were taken at intervals and the concentration of both ions of the salt in the samples determined. A quantity of tissue was removed with each sample of solution so that the proportion of tissue to solution remained the same. In the majority of the experiments of this type the atmosphere in the bottles was kept in connexion with the outer air by means of a hole, 1 cm. in diameter, in the stopper of the bottle, while the

bottles were kept continuously shaken by a mechanical shaking device. In addition, experiments were carried out on the absorption of manganese chloride in which (a) the bottles containing the tissue and solution were continuously shaken, but in which the stoppers were not perforated, and (b) the solutions were aerated intermittently. In other experiments manganese chloride was added daily so as to bring the concentration of the solution up to the original one of 0.001 M.

In further experiments with manganese chloride a different procedure was followed. The tissue was initially introduced into the solutions after preparation in the standard way, but after about twenty-four hours the solution was poured off and analysed for manganese and chloride while the tissue was transferred to a fresh quantity of solution equal in volume and concentration to the original solution. The same operation was repeated generally daily, in some cases for three weeks. In these experiments each bottle contained 75 discs and 150 ml. of solution, so that the proportion of tissue to solution was higher than in those in which the solution remained unchanged. In yet other experiments in which the solutions were renewed daily, a continuous current of air was passed through the solution. In these experiments, in order to avoid the error introduced into the estimation of the quantity of ions absorbed owing to evaporation of water, the tissue, after removal of the solution each day, was rapidly rinsed with distilled water, the latter added to the removed solution, and the whole made up to constant volume. The manganese and chloride were then determined in the resulting solutions.

All experiments were carried out at a temperature of 21° C. maintained by a 'sun-vic' control.

Manganese was estimated by means of the polarograph. The determination of manganese in this way has been described by Březina (1925) and Prajzler (1931), while the method has been used by Hamamoto (1934) for the determination of this element in animal tissue, and by Laine (1934) in his work on the absorption of electrolytes by the roots of *Phaseolus multiflorus*. It may be noted that Laine checked his results obtained polarographically with determinations by a colorimetric method and obtained good agreement between the two methods. Laine, accepting a statement of Kemula (1931) and others, assumes that the error in the polarographic determination of manganese may amount to 5 per cent., but our own experience is that with careful pipetting and temperature control during the measurement the error is much less than this, and should not generally exceed 1 or 2 per cent. of the concentration of the manganese in the original solution except in very low dilutions. In all determinations of manganese in solutions of the chloride and nitrate we used barium chloride as a ground liquid, the solutions polarographed being actually 5 ml. manganese salt solution + 5 ml. 0.05 M barium chloride. The samples from manganese salts of concentrations initially 0.0002 M and 0.001 M were used as sampled, while those from stronger solutions were diluted to this level before the determination of the manganese.

Each determination was made in duplicate. Measurement of the polarograms obtained with standard solutions showed that the relationship between concentration of manganese and height of 'wave' is linear.

Chloride was determined polarographically with the dropping mercury anode (Revenda, 1934), a method which we had found satisfactory in determining the absorption of chloride from potassium chloride solutions. Here also stronger solutions were diluted to the 0.001 M level before polarographing, and potassium nitrate was used as a ground liquid.

The determination of sulphate presented some difficulty. In our work on the absorption of potassium salts we estimated sulphate by a titration method involving the use of tetrahydroxyquinone as adsorption indicator (Schroeder, 1933). With potassium sulphate this was found to work fairly well, although in our experience the end point in the titration is never sharp. With manganese sulphate we found the difficulty of judging the end point even greater than with potassium sulphate. We therefore tried to determine sulphate by means of the polarograph. The procedure we finally adopted consisted in adding to 4 ml. of manganese sulphate solution (not stronger than 0.001 M) 5 ml. of 0.001 M barium chloride solution and 2 ml. of 0.1 M tetraethylammonium chloride and allowing the resulting mixture to stand for at least twenty hours for the precipitate of barium sulphate to settle. The clear liquid containing all the manganese and the rest of the barium was now polarographed and a polarogram obtained giving 'waves' for both manganese and barium. The height of the barium wave depends on the quantity of sulphate originally present in the mixture, the height of the barium wave being reduced in proportion to the amount of that ion removed from solution as barium sulphate. Calibration was effected by obtaining the waves for a number of solutions of manganese sulphate ranging in concentration from 0.00025 M to 0.001 M.

The agreement between determinations of sulphate in this way and those obtained by the use of the adsorption indicator was not good, and we consider that, with manganese sulphate, the polarographic determinations are the more reliable. A comparison of sulphate estimations by the two methods is given in Table V.

Nitrate was determined polarographically, as in the case of potassium nitrate, in the way described by Tokuoka and Růžicka (1934), in which lanthanum chloride is used as ground liquid.

All polarographic determinations of manganese, sulphate, and nitrate were carried out in an atmosphere of hydrogen.

## EXPERIMENTAL RESULTS

### *A. Absorption from Unchanged Solutions*

#### *1. Manganese chloride.*

Three series of experiments were carried out with manganese chloride in which samples from the same solution were taken out and analysed. The

three series of experiments were carried out on similar lines except that in one the standard technique was followed, that is, the bottles containing tissue and solution were connected with the outer air by a hole in the stopper and were continuously shaken, in the second the bottles were also continuously shaken but the stoppers were without the hole, while in the third the bottles were not shaken but the liquid in each bottle was aerated by a current of air for a period of twelve minutes in every hour by means of a pump brought into action at regular intervals by a time switch.

In the first series of experiments solutions of three concentrations, namely, 0.02 M, 0.004 M, and 0.001 M, were used. The course of absorption of the two ions as determined from the change in concentration of the external solutions is shown in Table 1. As in the previous paper in this series, all values are given as proportions of the original concentration of the external solution.

TABLE I

*Absorption of Manganese Chloride by Carrot Root Tissue*

Initial concentration.	Time (hours).	Absorption.	
		Mn.	Cl.
0.02 M	0.4	0.06	0.00
	2.67	0.04	0.01
	8.67	0.07	0.02
	19.6	0.05	0.03
	32.9	0.08	0.03
	50.5	0.07	0.01
	74.4	0.12	0.02
	93.2	0.12	0.05
0.004 M	0.33	0.07	0.03
	2.6	0.15	0.02
	8.4	0.20	0.05
	19.3	0.22	0.11
	32.6	0.20	0.13
	50.25	0.25	0.15
	74.2	0.32	0.17
	92.9	0.51	0.21
0.001 M	0.5	0.32	0.10
	2.67	0.51	0.11
	8.2	0.58	0.19
	19.2	0.63	0.28
	32.4	0.68	0.40
	50.1	0.74	0.46
	74.0	0.80	0.63
	92.75	0.83	0.70

An inspection of these numbers and of the curves of Figs. 1 and 2, in which the results are shown graphically, shows that whereas from the weakest solution used a considerable absorption of both kation and anion took place during the four days over which the experiment was continued, relatively little took place from the strongest solution, the amount of chloride amounting to only a few per cent., while the amount of manganese absorbed was not

much greater. The increase in relative absorption with fall of concentration is much more marked with manganese chloride than with potassium chloride. This will at once be evident if the results summarized in Table I of this paper are compared with those recorded in Table III of the previous paper of

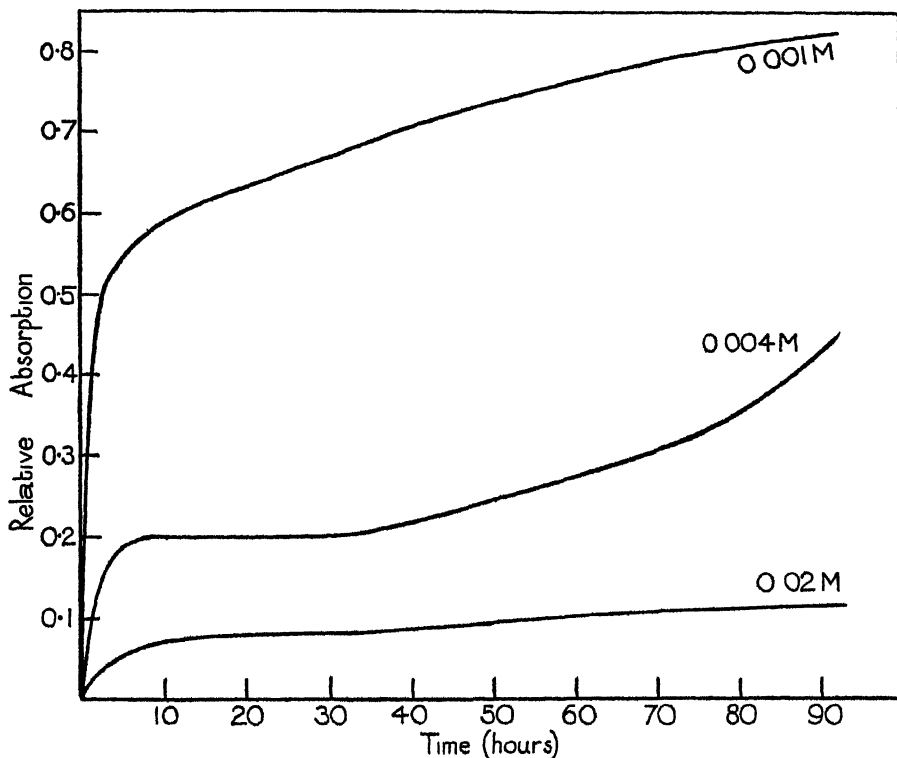


FIG. 1. Curves showing the course of absorption of manganese by carrot tissue from solutions of manganese chloride having initial concentrations of 0.02 M, 0.004 M, and 0.001 M.

this series, in which the results of a similar experiment with potassium chloride are given. From a 0.02 M solution of the latter salt the absorption of kation and anion over about four days amounted to 37 and 36 per cent. respectively of the total quantity present in the original solution as compared with 12 and 5 per cent. respectively from a 0.02 M solution of manganese chloride in the same time.

The course of absorption of manganese from its chloride is in striking contrast to the absorption of potassium from potassium chloride. With the latter salt absorption of the kation is slow at first and, indeed, there is often an exosmosis of potassium from the tissue into the external solution. With manganese chloride, on the other hand, there is a rapid initial absorption of the kation which, in the experiment recorded in Table I, amounted to half

of the original manganese content of a 0.001 M solution in the first 2.67 hours. By eight hours after the commencement of absorption the intake of manganese in the two higher concentrations had reached a position of approximate, though temporary, equilibrium, followed after some thirty or forty hours by

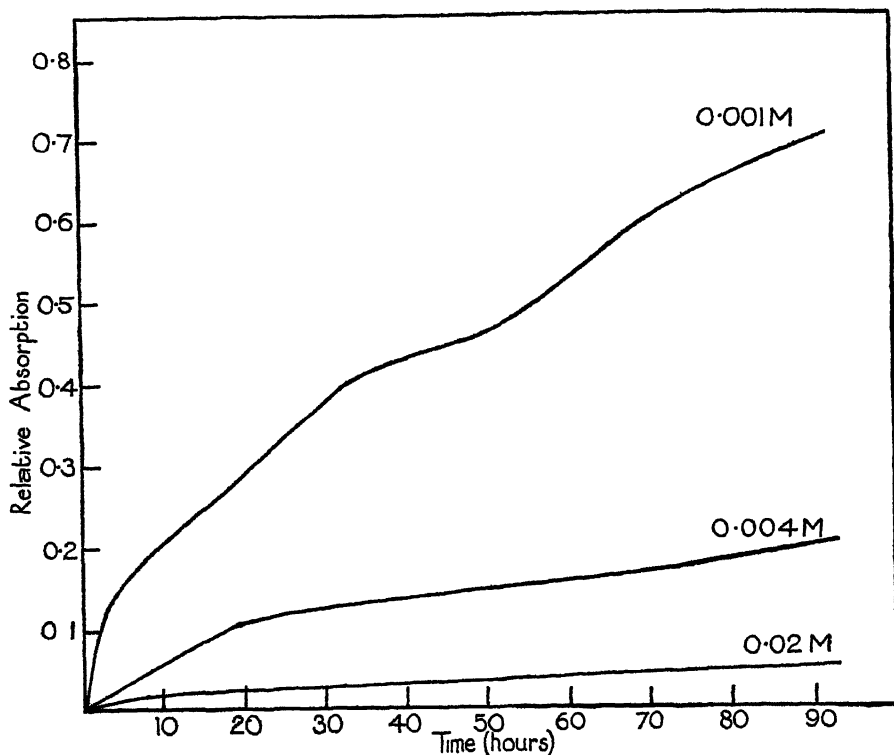


FIG. 2. Curves showing the course of absorption of chloride by carrot tissue from solutions of manganese chloride having initial concentrations of 0.02 M, 0.004 M, and 0.001 M.

further absorption which proceeded at a comparatively slow rate. In the lowest concentration the absorption appears to follow a continuous course, but even so from an inspection of Fig. 1 it will be seen that in this concentration also the course of absorption can be regarded as comprising a short initial phase of extremely rapid absorption followed by a long period of slower absorption. The slowness of the intake during this latter period may, however, in part be due to the low concentration which the manganese of the external solution has reached by this time.

The initial absorption of manganese greatly exceeded that of chloride in all concentrations, and in this respect also the results stand in marked contrast to those obtained with potassium chloride. At all stages in absorption the intake of the kation has exceeded that of the anion. The absorption of chloride

appears to follow a more regular course than that of manganese, and there is no indication of the attainment of a position of temporary equilibrium after about eight hours.

In a second experiment with manganese chloride the range of concentrations used was lower than in the former experiment, the actual concentrations being 0.005 M, 0.001 M, and 0.0002 M. In this experiment the stoppers of the bottles were not provided with holes, so that contact of the atmosphere in the bottle with the outside air was only made when samples of the solution were taken for analysis. But as the results tabulated in Table II show, the course of absorption of the two ions was similar to that observed in the experiment in which the atmosphere in the bottle was in constant connexion with the outer air.

TABLE II

*Absorption of Manganese Chloride by Carrot Root Tissue*

Initial concentration.	Time (hours).	Absorption.	
		Mn.	Cl.
0.005 M	0.40	0.08	0.07
	4.5	0.17	0.08
	23.1	0.22	0.15
	47.33	0.21	0.19
	73.2	0.30	0.21
0.001 M	0.33	0.17	0.09
	2.4	0.52	0.10
	5.5	0.55	0.19
	23.33	0.55	0.27
	47.6	0.66	0.33
	73.33	0.88	0.54
0.0002 M	0.4	0.30	0.32
	2.8	0.85	0.33
	6.0	0.85	0.36
	23.7	0.86	0.66
	48.0	0.90	—
	74.9	—	0.88

The rapid absorption of manganese from the lowest concentration is very noticeable.

A third experiment in which the same concentrations were used differed from the previous experiment in that the solutions were not shaken but were aerated intermittently. The course of absorption was, however, practically identical with that in the previous experiment, as the results collected in Table III show.

## 2. *Manganese nitrate.*

The absorption of manganese nitrate by carrot tissue was examined by the standard method in which the tissue was continuously shaken and the atmosphere in the bottles kept in constant connexion with the outside air. The



concentrations used were 0.02 M, 0.005 M, and 0.001 M. The results are summarized in Table IV and shown graphically in Figs. 3 and 4.

TABLE III

*Absorption of Manganese Chloride by Carrot Root Tissue*

Initial concentration.	Time (hours).	Absorption.	
		Mn.	Cl.
0.005 M	0.4	0.08	0.06
	4.5	0.13	0.09
	23.1	0.18	0.10
	47.3	0.25	0.15
	73.2	0.27	0.21
0.001 M	0.33	0.11	0.09
	2.4	0.17	0.10
	5.5	0.52	0.10
	23.3	0.54	0.22
	47.6	0.62	0.19
0.0002 M	73.3	0.70	0.42
	0.4	0.30	0.19
	2.8	0.75	0.30
	6.0	0.80	0.33
	23.7	0.87	0.38
	48.0	0.90	0.68
	74.9	—	0.88

TABLE IV

*Absorption of Manganese Nitrate by Carrot Root Tissue*

Initial concentration.	Time (hours).	Absorption.	
		Mn.	NO <sub>3</sub> .
0.02 M	0.4	0.06	0.02
	7.0	0.10	0.00
	19.6	0.09	0.01
	32.5	0.11	0.01
	50.1	0.10	0.01
	74.6	0.15	0.02
	98.2	0.12	0.04
	120.0	0.20	0.03
0.005 M	0.4	0.09	0.00
	7.1	0.21	0.01
	19.6	0.22	0.04
	32.3	0.22	0.07
	50.1	0.26	0.16
	74.6	0.35	0.18
	98.2	0.36	0.19
	120.0	0.38	0.23
0.001 M	0.4	0.29	0.04
	7.1	0.55	0.12
	19.7	0.55	0.27
	32.3	0.61	0.39
	50.1	0.66	0.50
	74.6	0.80	0.78
	98.2	0.87	0.99
	120.0	0.91	0.99

The general course of absorption of manganese nitrate shows the same characteristics as that of the chloride. There is the same very marked effect of concentration, the same rapid initial absorption of manganese reaching a temporary condition of approximate equilibrium by about seven hours, and

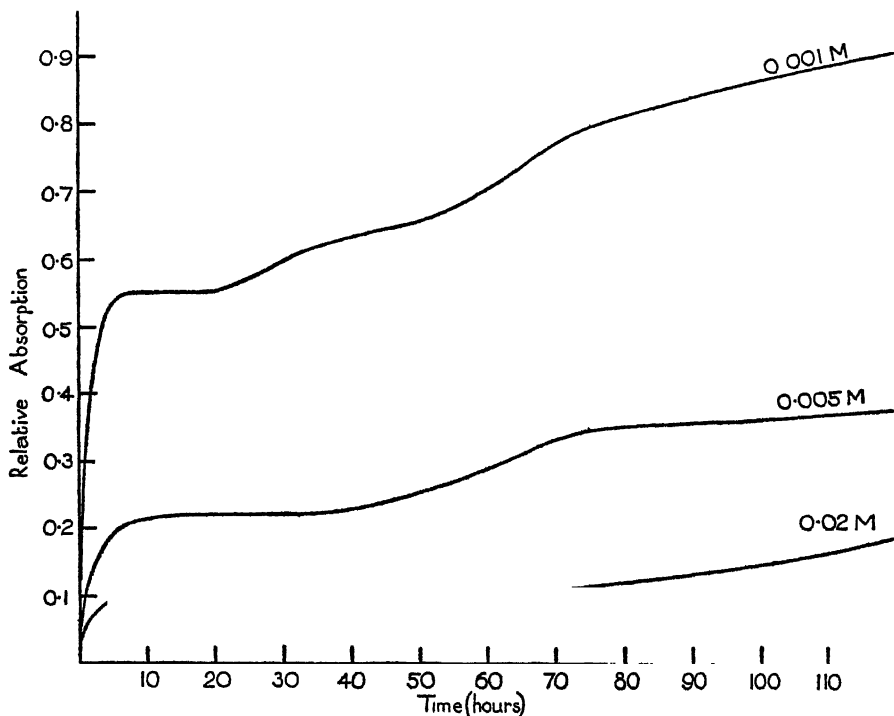


FIG. 3. Curves showing the course of absorption of manganese by carrot tissue from solutions of manganese nitrate having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

the same subsequent slow absorption. The course of absorption of the anion is also similar to that of chloride from manganese chloride. In the highest concentration, 0.02 M, the amount of anion absorbed is only a few per cent. of that present in the external solution; indeed, the values obtained for the nitrate absorbed are scarcely outside the experimental error of the determination and it is quite clear that in this concentration, as well as in the 0.005 M solution, the absorption of nitrate is less than that of manganese. From the weakest solution, however, the absorption of nitrate, although at first very much slower than that of manganese, ultimately surpasses that of the kation, so that after four days practically all the nitrate had been removed from the solution by the tissue.

### 3. *Manganese sulphate.*

In the experiments on the absorption of the ions of manganese sulphate by

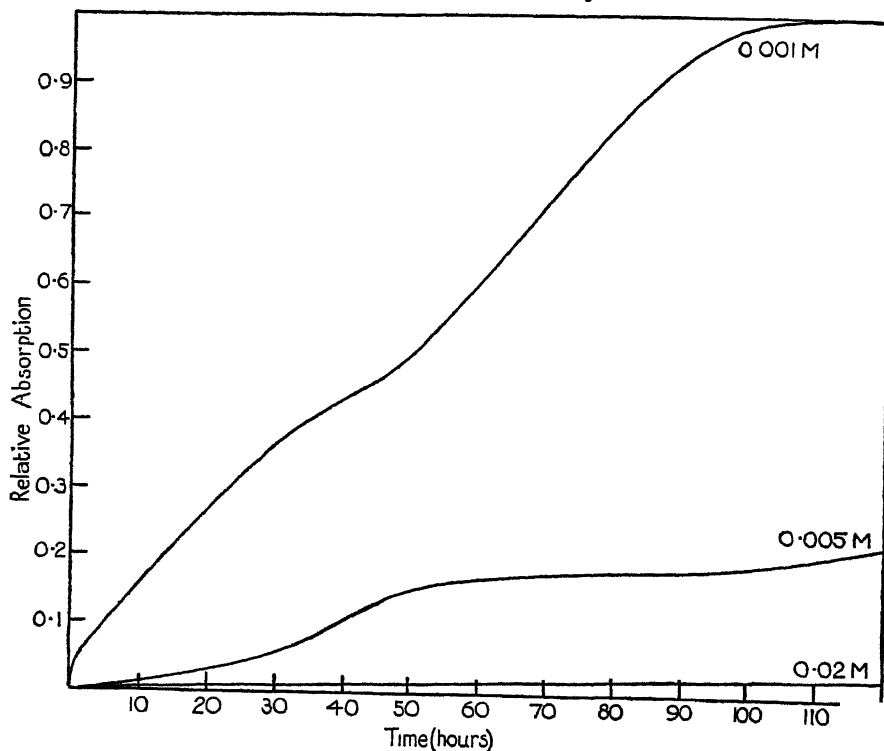


FIG. 4. Curves showing the course of absorption of nitrate by carrot tissue from solutions of manganese nitrate having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

TABLE V

*Absorption of Manganese Sulphate by Carrot Root Tissue*

Initial concentration.	Time (hours).	Mn.	Absorption. SO <sub>4</sub> .	
			by polarograph.	by titration.
0.02 M	0.8	0.06	0.02	0.06
	19.3	0.08	0.00	0.03
	52.6	0.11	0.02	0.03
	92.8	0.13	0.02	0.03
	138.7	0.14	0.02	0.05
	188.25	0.15	0.14	0.10
0.005 M	0.8	0.16	0.06	—0.03
	19.2	0.24	0.09	0.03
	52.8	0.27	0.10	0.03
	92.7	0.32	—	0.10
	138.7	0.32	0.12	0.17
	188.1	0.32	0.19	0.23
0.001 M	0.7	0.41	0.08	0.10
	19.2	0.57	0.14	0.10
	52.8	0.60	0.24	0.20
	92.6	0.64	0.39	0.33
	138.6	0.72	0.50	0.56
	188.0	0.75	0.54	—

carrot root tissue solutions of the same three concentrations were used, namely, 0.02 M, 0.005 M, and 0.001 M. The course of absorption in the first experiment is shown by the data of Table V.

The results of a second series of experiments on the absorption of manganese sulphate are shown in Table VI. Here the determination of sulphate was made polarographically only. The course of absorption of the manganese is shown graphically in Fig. 5.

TABLE VI

*Absorption of Manganese Sulphate by Carrot Root Tissue*

Initial concentration.	Time (hours).	Absorption.	
		Mn.	SO <sub>4</sub> .
0.02 M	0.4	0.04	0.00
	3.1	0.07	—
	9.2	0.07	—
	19.6	0.07	—0.02
	33.7	0.10	—
	52.3	0.08	0.00
	73.6	0.11	—
	104.3	0.11	0.02
0.005 M	0.4	0.11	—0.01
	3.2	0.17	0.00
	9.3	0.18	0.00
	19.7	0.18	0.02
	33.8	0.20	0.05
	52.4	0.20	0.05
	73.7	0.21	0.05
	104.3	0.26	0.05
0.001 M	0.5	0.07	0.00
	3.3	0.48	0.00
	9.3	0.51	0.03
	19.8	0.51	0.06
	33.8	0.53	0.09
	52.5	0.54	0.13
	73.8	0.57	0.21
	104.4	0.59	0.29

After absorption had proceeded from these solutions for about 104 hours the discs from the weakest solution were transferred to a fresh solution of 0.001 M concentration and the course of absorption of the two ions of the salt followed for another 136 hours. The results are summarized in Table VII.

Inspection of the numbers given in Tables V and VI show that the course of absorption of the ions of manganese sulphate is very similar to that of the other manganese salts examined, except that the rate of absorption of the ions of the sulphate is less, both as regards kation and anion, than those of the chloride and nitrate. The absorption of the kation again shows the initial phase of rapid absorption to a position of temporary equilibrium within nine hours followed after some hours by a slow absorption which continued until the experiment was stopped. The absorption of the sulphate ion from

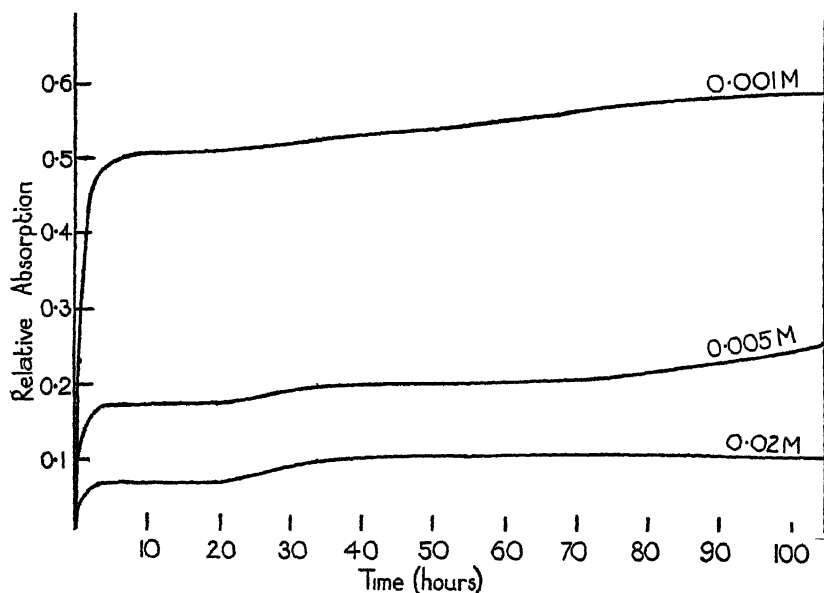


FIG. 5. Curves showing the course of absorption of manganese by carrot tissue from solutions of manganese sulphate having initial concentrations of 0.02 M, 0.005 M, and 0.001 M.

TABLE VII

*Further Absorption of Manganese Sulphate from a 0.001 M Solution after Preliminary Absorption for 104 Hours*

Time in hours after transference to fresh solution.	Further absorption.	
	Mn.	SO <sub>4</sub> .
0.5	0.195	0.05
16.9	0.32	0.11
36.3	0.34	0.15
61.6	0.41	0.21
91.9	0.46	0.30
114.7	0.56	0.36
136.6	0.57	—

the strongest solution employed was negligible and still very slight from the 0.005 M solution. From the 0.001 M solution, however, the absorption, although very much less than that of either chloride or nitrate, proceeded at a measurable rate throughout the experiment so that after four days more than a quarter of the sulphate originally present in the solution had been removed by the tissue. The observed rate of absorption of sulphate from a 0.001 M solution of manganese sulphate was actually about the same as that observed from a solution of potassium sulphate of the same concentration. With the higher concentrations, however, the absorption of the anion is

strikingly less from manganese sulphate than from potassium sulphate solutions of the same concentrations.

*B. The Effect of Concentration of the Salt on the First Phase of its Absorption*

The similarity of the effect of concentration of the salt on its absorption to the adsorption equation has been noted several times (Stiles and Kidd, 1919; Stiles, 1924; Laine, 1934). We have pointed out in the first paper of this series that this similarity would seem to refer to the first phase of absorption only. It has already been shown in the present paper that the first phase of rapid absorption of manganese is complete within about seven hours, and it is thus of interest to examine the effect of concentration on the amount of manganese absorbed from various solutions after this time. In Table VIII are collected the relevant data bearing on this point yielded by the experiments already described. The external concentrations are given as milli-equivalents of ion per litre, and the internal concentration as milli-equivalents of ion per 1,000 c.c. water in the tissue. This is, of course, an average value for the whole tissue since we have no information regarding the distribution of manganese in the tissue after its absorption. The logarithms to base 10 of the final internal and external concentrations are also given for the sake of later discussion, as well as the absorption ratios, that is, the ratios of final internal to final external concentration in each case.

TABLE VIII

*Effect of Concentration on Absorption of Manganese by Carrot Root during the First Phase of Absorption*

Salt and absorption period.	Initial external concentration.	Final external concentration (E).	Final internal concentration (I).	Log E.	Log I.	Absorption ratio (I/E).
MnCl <sub>2</sub>	20	18.6	16.24	1.2695	1.211	0.87
	4	3.20	9.25	0.505	0.966	2.89
	1	0.42	6.71	1.623	0.827	16.0
Mn(NO <sub>3</sub> ) <sub>2</sub>	20	18.4	18.6	1.265	1.2695	1.01
	5	3.95	12.1	0.597	1.083	3.06
	1	0.45	6.36	1.653	0.8035	14.15
MnSO <sub>4</sub>	20	18.6	16.2	1.2695	1.211	0.87
	5	4.125	10.1	0.615	1.004	2.45
	1	0.49	5.90	1.690	0.771	12.05

A further examination of this point was made with manganese chloride in an experiment in which five different concentrations of this salt were employed, varying from 0.01 M to 0.0005 M. The position of absorption after 7.5 hours is shown in Table IX. All concentrations are given in milli-equivalents per litre, as in the previous table.

TABLE IX

*Effect of Concentration on the Initial Phase of Absorption of Manganese from Manganese Chloride by Carrot Root*

Initial external concentration.	Final external concentration (E).	Final internal concentration (I).	Log E.	Log I.	Absorption ratio (I/E).
10	8.72	14.85	0.9405	1.1718	1.70
5	3.90	12.77	0.5911	1.1062	3.27
2	1.254	8.65	0.0983	0.9372	6.90
1	0.462	6.24	1.6646	0.7953	13.5
0.5	0.1655	3.88	1.2188	0.5888	23.4

The effect of concentration on the absorption of manganese thus exhibits the same characteristic that has been already observed with many other ions, namely, the increase in the amount absorbed, relative to the concentration, with decreasing concentration. The degree of concordance of the absorption-concentration relation with the adsorption equation is indicated by the graphs in Fig. 6, in which the logarithms of final internal concentration are plotted against those of final external concentration. It will be observed that the relationship between these quantities is approximately linear, and we may therefore conclude that the relationship between concentration of manganese and the amount of it absorbed during the first phase of absorption is approximately given by the adsorption equation

$$\log I = k \log E + C \text{ or } I = CE^k,$$

where  $I$  and  $E$  are the final internal and external concentrations and  $C$  and  $k$  are constants. This relationship has also been observed by Laine to hold for the absorption of manganese by cut roots of *Phaseolus multiflorus*.

In connexion with this apparent similarity of kation absorption with adsorption we would call attention to the experiment with manganese sulphate in which, after absorption had proceeded from a solution for more than four days, the tissue was transferred to a fresh solution. As Tables VI and VII show, the course of absorption from the new solution is practically a repetition, though at a somewhat lower level, of that from the original solution. There is again the initial phase of rapid absorption followed by a continued slow intake. Now if the amount of absorption during the initial phase is determined solely by concentration in the way we have indicated, we should expect a further rapid intake of manganese on transference to the fresh solution since the external concentration is thereby raised from 0.41 to 1.0 milli-equivalents per litre. But if we calculate from the adsorption equation the extra amount of manganese which should be absorbed on this assumption we find this amount is considerably less than that which is actually taken in. Thus, referring to Table VII, it will be seen that the manganese absorbed in the initial phase after transference to the new solution amounted to 0.32,

corresponding to an increase in the internal concentration of 3.71 milli-equivalents per 1,000 c.c. of water in the tissues, while the concentration of the external solution at the end of this phase was  $0.68 \times 10^{-3}$  M. The internal concentration of manganese corresponding to this, according to the adsorption relation, is found by interpolation from the numbers given in Table VIII to

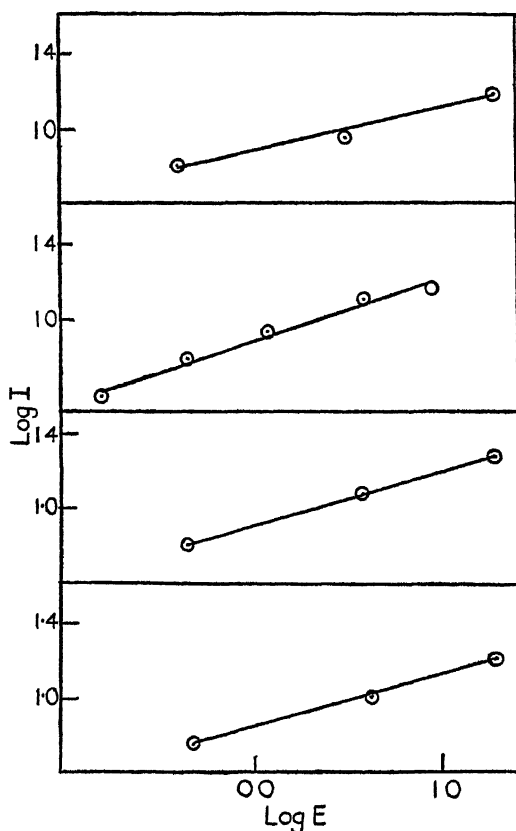


FIG. 6. The relationship between final internal and external concentrations of manganese after the first phase of absorption by carrot tissue from solutions of various manganese salts. The graphs, from above downwards refer to the chloride (Table I), chloride (Table IX), nitrate and sulphate.

be 6.64 milli-equivalents per 1,000 c.c. of water in the tissues. Now the manganese absorbed during the first phase from the first solution corresponded to an internal concentration of 5.90 milli-equivalents per 1,000 c.c. of water in the tissues, so that the total amount of manganese taken in by the initial process after transference to the fresh solution, and including that taken in from the first solution, thus amounted to  $5.90 + 3.71$  or 9.61 milli-equivalents of manganese per 1,000 c.c. of water in the tissues, a quantity very much in excess of the theoretical value of 6.64. Or, put in another way, assuming the initial phase of absorption to be determined by adsorption or some analogous



process, we should expect the additional manganese absorbed on this account to be that amount which would raise the internal concentration by 0.74 milliequivalents per 1,000 c.c. of water in the tissues; actually it is five times this amount.

It would therefore appear that the amount of kation absorbed during the first phase of absorption must be determined by some other factor in addition to the concentration of the kation in the external solution, a factor which is affected by the transference of the tissue to fresh solution. The only obvious change effected by this transference, apart from the increase in concentration of the manganese salt, is the removal of any solutes which have passed out from the tissue into the external medium, and this would suggest that the initial absorption is at least in part related to the exosmosis of ions from the tissue. That this must be so is also indicated by the great difference in the quantities of kation and anion of the manganese salt absorbed during the initial phase. For maintenance of equality of positive and negative ionic charges in the external solution there must either be an exchange of kations from the tissue with the excess manganese absorbed, or the excess of manganese must enter the tissue accompanied by hydroxyl ions from water which would result in the external medium becoming acid. Determinations of the pH of the external solutions show that there is no such production of hydrogen ions in these solutions, and we must therefore conclude that the initial phase of absorption is intimately connected with ionic exchange between the tissue and external medium. To help towards a further analysis of this question the experiments described in the next section of this paper were accordingly carried out.

### *C. The Course of Absorption of the Ions of Manganese Chloride when the External Solution is Renewed Daily*

The routine observed in the experiments recorded in this section of this paper has been indicated earlier. In the first series of experiments two concentrations of manganese chloride were used, namely, 0.005 M and 0.001 M. The quantities were determined of manganese and chloride absorbed on successive days from solutions having these respective initial concentrations at the beginning of each daily period.

In Table X are shown the results of a pair of experiments in which the weaker solution was employed. The absorption is again shown as the proportion of the ion originally present in the solution taken in during each period.

The factor with which these numbers have to be multiplied to give the increase in concentration of the ions during each period is 6.74.

It will be observed that absorption is still taking place three weeks after the beginning of the experiment. For the first eight or nine days the amount of manganese absorbed during each daily period is less than during the preceding period, and the same is true on the whole for the absorption of chloride.

TABLE X

*Absorption of Manganese Chloride from 0.001 M Solutions*

Period.	Length of period (hours).	Absorption.			
		Experiment 1.		Experiment 2.	
		Mn.	Cl.	Mn.	Cl.
1	26.0	0.72	0.45	0.73	0.42
2	25.25	0.57	0.44	0.57	0.43
3	23.8	0.42	0.40	0.42	0.34
4	23.25	0.32	0.31	0.365	0.32
5	23.2	0.26	0.34	0.29	0.21
6	23.5	0.22	0.26	0.24	0.17
7	23.6	0.18	0.215	0.22	0.15
8	47.2	0.31	0.30	0.38	0.34
9	24.9	0.22	0.19	0.27	0.20
10	24.1	0.30	0.16	0.305	0.25
11	24.0	0.12	0.09	0.09	0.13
12	23.8	0.12	0.15	0.12	0.19
13	23.9	0.125	—0.03	—0.02	—0.05
14	41.3	0.195	0.28	0.24	0.17
15	30.6	—0.02	—0.06	0.01	—0.01
16	24.0	0.06	0.10	0.065	0.16
17	24.1	0.09	0.15	0.09	0.17
18	24.2	0.08	0.13	0.06	0.11

Indeed, after the first two or three days, when the absorption of kation is markedly in excess of that of the anion, the absorption of the two ions, though not in strictly equivalent quantities, is of the same order. After nine days the absorption shows daily fluctuations which are unexplained but which may be due to local damage to a few cells of the tissue, although the fact that these variations appear at the same time in both experiments militates against this idea.

While the quantities of ions absorbed are less on successive days, it is nevertheless remarkable that the quantities absorbed are considerable even after eight or nine days. Indeed, these results, like those already recorded with manganese sulphate, indicate conclusively that the initial phase of kation absorption must be largely determined by ionic interchange. We have again calculated what should be the amount of manganese absorbed for the 0.001 M during the first week if this were solely determinable from the adsorption equation. The results of these calculations are shown in Table XI, where the total

TABLE XI

*Total Absorption of Manganese up to the End of each Period*

Period.	Calculated on basis of adsorption.	Actual absorption.
1	0.725	0.725
2	0.84	1.295
3	0.92	1.715
4	0.96	2.06
5	0.99	2.33
6	1.01	2.56
7	1.02	2.76

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amounts of manganese which should be absorbed on this basis up to the end of each daily period are compared with the quantities actually absorbed.

It will be observed that the actual absorption very considerably exceeds that which would be expected if the amount were determined by the concentration of the solution, and, as in the case of the sulphate, this must be ascribed to the removal of ions which have passed out from the tissue and which are removed when the solution is renewed. There will thus result a further exchange of ions with each renewal of the external solution. Since this will result in a continued diminution of the exchangeable ions in the tissue there will be a corresponding day-to-day diminution in the amount of manganese absorbed in this way. This is certainly the case for absorption from 0.001 M solutions for the first seven days, after which period the same trend appears to continue although the results are not so regular.

The course of absorption when a stronger solution of 0.005 M concentration is used is strikingly different. The results of an experiment carried out in duplicate with manganese chloride in this concentration and in which the solutions were renewed (generally) daily are shown in Table XII. It will be seen that in this case absorption is negligible after the fourth day. Nevertheless, the amounts of manganese absorbed during the second and third daily periods are again greater than would be the case if concentration of manganese alone was determining the amount of absorption according to the adsorption equation.

TABLE XII

*Absorption of Manganese Chloride from 0.005 M Solutions*

Period.	Length of period (hours).	Absorption.			
		Experiment 1.		Experiment 2.	
		Mn.	Cl.	Mn.	Cl.
1	26.0	0.33	0.05	0.32	0.05
2	25.25	0.17	0.01	0.11	0.00
3	23.8	0.09	0.01	0.05	-0.02
4	23.25	0.08	0.00	0.01	0.01
5	23.2	0.03	—	-0.02	—
6	23.5	0.03	—	-0.02	—
7	23.6	0.00	—	-0.02	—
8	47.2	-0.01	—	0.00	—
9	24.9	-0.03	—	0.00	—
10	24.1	0.00	—	-0.02	—

The absorption is again shown as the proportion of the ion originally present in the external solution absorbed during each period. If these numbers are multiplied by 33.7 they give the increase in concentration of the ions in the tissue during each period stated as milligram-ions per 1,000 c.c. of water in the tissue.

Confirmation of the results was afforded by a series of experiments in which absorption of manganese chloride by samples of the same batch of tissue was examined under three different sets of conditions. In the first of these the

solutions were not changed, but at the end of each day enough manganese chloride was added to bring the concentration up to approximately the original one of 0.001 M. In the second the conditions were the same as those in the experiments described earlier in this section of this paper, that is, the solutions were renewed daily. In the third the solutions were also renewed daily, but a constant stream of air was passed through the solutions and the routine described earlier for this type of experiment was observed. The quantitative relations of tissue and solution were the same in each case and two experiments of each kind were carried out. The results of these experiments with 0.001 M manganese chloride are summarized in Tables XIII and XIV, which show respectively the amounts of manganese and chloride absorbed. These quantities are again given in terms of the proportion of the original quantity of ion present taken in during each daily period. In these experiments each period was a day of twenty-four hours.

In each experiment the concentration of the external solution at the

TABLE XIII

*Absorption of Manganese from 0.001 M Manganese Chloride under various Conditions*

Daily period.	Absorption of manganese.					
	Solutions unchanged, concentration made up daily.		Solutions changed daily, unaerated.		Solutions changed daily, aerated.	
	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.
1	0.78	0.795	0.74	0.75	0.75	0.76
2	0.195	0.35	0.56	0.545	0.55	0.60
3	0.17	0.24	0.38	0.36	0.44	0.44
4	0.14	0.18	0.29	0.31	0.33	0.40
5	0.12	0.12	0.16	0.19	0.20	0.22
6	0.07	0.15	0.06	0.13	0.20	0.22
7	0.14	0.16	0.07	0.10	0.17	0.21

TABLE XIV

*Absorption of Chloride from 0.001 M Manganese Chloride under various Conditions*

Daily period.	Absorption of chloride.					
	Solutions unchanged, concentration made up daily.		Solutions changed daily, unaerated.		Solutions changed daily, aerated.	
	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.	Expt. 1.	Expt. 2.
1	0.37	0.38	0.30	0.28	0.40	0.32
2	0.24	0.31	0.36	0.31	0.26	0.28
3	0.23	0.27	0.23	0.25	0.205	0.23
4	0.17	0.21	0.24	0.275	0.12	0.18
5	0.09	0.23	0.20	0.22	0.13	0.14
6	0.14	0.07	0.12	0.14	0.11	0.14
7	0.14	0.15	0.13	0.14	0.13	0.14

beginning of each daily period was thus approximately the same, namely, 0.001 M. But in the series where the solutions were unchanged the absorption of manganese during the second, third, fourth, and fifth days was very definitely less than when the solutions were renewed. Thus it will be seen that the absorption of manganese was approximately the same from all the samples of tissue during the first day, but that during the second day the average absorption of manganese from the unchanged solution was only about half that which took place from the solutions which had been renewed. This state of affairs was much the same on the third and fourth days. On the fifth day, while absorption was still greater from the changed than from the unchanged solutions, the difference was not so great. After this the absorption from the unchanged solution is still less than from the changed aerated solutions, although more than the absorption from the changed unaerated solutions. The absorption of manganese from these last on the sixth and seventh days may, however, be exceptionally low, for the absorption on the corresponding days in the exactly similar experiment recorded earlier (see Table X) was much higher.

On the assumption that the initial phase of absorption of manganese is determined in greater part by ionic exchange, we should expect the excess absorption from changed solutions as compared with that from unchanged ones to be less in succeeding absorption periods, since renewal of the external solutions involves a continued lessening of the amount of exchangeable ions in the tissue.

Just as there is no evident first phase of rapid absorption of the anion, so markedly more chloride does not appear to be absorbed from the changed than from the unchanged solutions, although it should be noted that the daily initial chloride concentrations of the unchanged solutions are greater than that of 0.001 M manganese chloride since, owing to the excess absorption of manganese over chloride, making up the concentration of manganese to its original value by addition of manganese chloride involves making up the chloride to a higher concentration. However, having regard to the effect of concentration on absorption the amount of chloride absorbed will not have been increased by more than about 20 per cent. owing to the increase in concentration above that in the changed solutions. It seems, therefore, reasonable to conclude that ionic exchange plays at most a minor part in the absorption of chloride from manganese chloride.

Some further experiments in which solutions were changed daily have also been carried out on the absorption of manganese chloride from solutions of 0.005 M concentration. In two cases the solutions were continually aerated, in the third they were not. The results of these experiments are summarized in Table XV.

These results confirm those already recorded in Table XII and emphasize the rapid falling off in the quantities of both manganese and chloride absorbed from solutions of this concentration.

TABLE XV

*Absorption from 0.005 M Manganese Solutions Renewed Daily*

Absorption during each daily period.

Daily period.	Un-aerated.		Expt. 1.		Expt. 2.	
	Mn.	Cl.	Mn.	Cl.	Mn.	Cl.
1	0.36	0.10	0.33	0.06	0.34	0.075
2	0.21	0.09	0.13	0.00	0.135	0.01
3	0.15	0.07	0.085	0.01	0.085	0.00
4	0.12	0.06	0.08	0.05	0.065	0.03
5	0.12	0.05	0.03	0.04	0.04	0.00
6	0.05	0.04	—0.01	—0.01	0.02	0.00
7	0.025	0.04	0.02	0.01	0.05	0.05

DISCUSSION

1. *The course of absorption.*

The intake of manganese from unchanged solutions of the three salts used was found to follow a similar course from solutions of the same concentration. From dilute solutions, 0.001 M or less, there was a rapid initial absorption of manganese amounting after about eight hours to 50 per cent. or more of that originally present in the external solutions. From solutions of medium concentrations, 0.004 M or 0.005 M, there was also a rapid initial absorption of manganese, but only from 17 to 22 per cent. of the manganese originally present in the external solution was absorbed. From the strongest solutions employed comparatively little manganese was absorbed even after four or five days, but at least half of the total kation absorbed after this time had elapsed was taken in during the first eight hours.

This initial period of rapid kation absorption was usually followed by a period of about twenty-four hours in which little or no absorption of manganese took place, after which further absorption proceeded slowly until the end of the experiment after another two or three days. Although an exact comparison of the rates of absorption of manganese from different salts cannot be made from the results here recorded, as the experiments with different salts were carried out with different samples of tissue, we can nevertheless conclude that the amounts of manganese absorbed from the chloride and nitrate solutions of the same concentration were about the same after the same time, while the amount absorbed from the sulphate was definitely less than from the salts of the monobasic mineral acids.

We may thus conclude that the absorption of manganese from an unchanged solution follows a two-phase course, an initial phase of rapid absorption which reaches a position of approximate and temporary equilibrium followed by a period of slow absorption which continues at any rate until the fourth or fifth day from the first immersion of the tissue in the solution.

While the course of absorption of manganese by carrot root tissue is similar to that of potassium from the corresponding salts (Stiles and Skelding, 1940)

in so far as at least two phases in the absorption can be made out, there are certain definite differences. In the first place there is no observable initial rapid absorption of potassium, a difference probably related to the presence of much potassium and very little manganese in the tissue. Secondly, the end of the period of rapid absorption occurs considerably earlier with manganese than with potassium, a difference which may perhaps also be related to the presence of potassium in the tissue and the initial period of exosmosis of this ion. Thirdly, the rate of absorption of manganese during the later phase is considerably slower than that of potassium from the corresponding salt in the same concentration, a difference which is perhaps related to the valency of the ion or to the fact that potassium is a major nutrient while manganese is a micro-nutrient. A fourth difference is in the effect of concentration, to which reference will be made later.

There was no marked biphasic character observable in general in the course of absorption of any of the anions, although one or two of the curves for absorption of chloride from manganese chloride might be regarded as showing such a course. There is certainly no rapid initial absorption of the anion at all similar to that of the kation, but subsequent absorption of anion may be more rapid than that of the kation, at any rate from the dilute solutions of manganese chloride and manganese nitrate used, with the result that the total intake of anion may approach, or even exceed, that of the kation after absorption has proceeded for some days.

## 2. *The unequal absorption of the two ions of manganese salts.*

The excess absorption of manganese over that of the anion is a marked feature of the initial phase of absorption of all the manganese salts examined. In this respect the latter differ markedly from potassium salts, in which any excess absorption of kation over anion is never very great and in which quite frequently the initial absorption of the anion of the salt may exceed that of the kation. This difference in behaviour is again probably related to the presence of a considerable amount of potassium and almost complete absence of manganese in the tissues. In this connexion it may be noted that a similar notable excess absorption of kation over anion was observed by Steward and Harrison (1939) in the absorption by potato tuber tissue of rubidium bromide from a 0.002 M solution, where also the tissue will initially have contained a negligible amount of the external kation. After the initial phase of absorption the difference in the rates of intake of the two ions becomes much less, but we should not consider ourselves justified in assuming as Steward and Harrison do for the absorption of rubidium bromide, that the two ions are absorbed at the same rate during the second phase. Indeed, the tendency seems to be for the rate of absorption of the anion to come to exceed that of the kation, so that the difference between the total amounts of the two ions absorbed becomes less with time. Actually in our experiment with 0.001 M manganese nitrate the total absorption of the anion finally exceeded that of the kation.

### 3. The effect of concentration.

We have shown that the effect of concentration on the total amount of manganese absorbed during the initial phase of absorption is approximately expressed by the adsorption equation

$$I = CE^k,$$

where  $I$  and  $E$  are the final internal and external concentrations of the manganese and  $C$  and  $k$  are constants. The same relation has not only been previously found for the absorption of other salts by storage tissues (cf. Stiles and Kidd, 1919; Stiles, 1924) but has also been found by Laine (1934) for the absorption of manganese by the excised roots of *Phaseolus multiflorus*. Laine found that in this case  $k$  had the value of about 0.3. It is therefore interesting to inquire into the values of this constant in our experiments on the absorption of manganese by storage tissues. These values are at once obtained from the graphs shown in Fig. 6. They are summarized in Table XVI.

TABLE XVI

*Effect of Concentration on the Initial Phase of Absorption of Manganese by Storage Tissues*

Salt.	$k$ .
Manganese chloride (Table I)	0.25
" " (Table IX)	0.34
" nitrate	0.30
" sulphate	0.29

It will thus be observed that the value of  $k$  is approximately the same for all the manganese salts examined and that it has also the same value for carrot root tissue and for the roots of *Phaseolus multiflorus*. So far, then, as our information at present allows, we can conclude that the effect of concentration on the intake of manganese is the same for different salts and different tissues. Admittedly the data are as yet too few to allow us to be dogmatic on the question.

It will be noted that the smaller the value of  $k$  the greater will be the effect of concentration in reducing the absorption of an ion relative to the concentration. It must be left for further work to show how far the value of  $k$  is determined by the nature of the ion.

We have already pointed out that although the relation between quantity of absorption during the first phase and concentration is given by the adsorption equation, adsorption cannot be the principal factor controlling this absorption and that ionic exchange must play an important part in this process. It is therefore of interest to examine whether the observed concentration effect can be explained by ionic interchange. The possibility of the absorption of ions apparently against a concentration gradient being conditioned by a Donnan equilibrium has often been pointed out. For such an equilibrium to be reached the tissues must contain a non-mobile ion balanced by a mobile



ion and the proteins of the tissues are generally supposed to provide such a system. There is evidence that the non-mobile ion is the anion.

Let us, then, for the sake of simplicity, suppose that our system at the beginning of an experiment consists of a quantity of external solution of volume  $V_e$  in which is immersed tissue with an effective volume of  $V_i$ . Let us further suppose that the external solution is that of a single salt with monovalent ions and that the quantity of each is  $a$ , so that the concentration of each is  $a/V_e$ . Let us also suppose that the immobile anion in the tissue is balanced by a quantity  $b$  of a univalent kation, the initial concentration of which is thus  $b/V_i$ . Then if the quantities of kation and anion absorbed by the tissue from the external solution are respectively  $x$  and  $y$ , at equilibrium we shall have the following relationships:

$$\frac{(a-x)(a-y)}{V_e^2} = \frac{xy}{V_i^2}$$

and

$$\frac{(a-y)(x-y)}{V_e^2} = \frac{y(b-x+y)}{V_i^2},$$

from which  $x$  and  $y$  can be determined if we know  $a$ ,  $b$ ,  $V_e$ , and  $V_i$ . Now in any experiment the only quantities which are definitely known are  $V_e$  and  $a$ . As regards  $V_i$ , the effective internal volume, we can find the amount of water in the tissue and as a first approximation assume the volume of this to be the internal volume of the tissue. It has, however, to be borne in mind that each cell of the tissue contains more than one phase and absorption of one ion may be limited mainly to one phase (cf. Briggs and Petrie, 1928). There is also the possibility that absorption may not be uniform throughout the tissue and may be largely confined to the outer region (cf. Steward, Wright, and Berry, 1932).

As regards  $b$  we are equally in the dark, for although the total quantity of various ions in the tissues are measurable, we do not know how these are distributed and the quantity we require to know is the quantity of kation, for example, balancing the immobile anion in the absorbing phase. For the sake of argument and simplicity let us assume that in experiments of the type we have employed the effective volume of the external solution is ten times that of the tissue, and that the amount of the kation in the tissue balancing the immobile anion is unity as compared with quantities of kation initially present in the external solution of 1, 4, and 20 respectively in three experiments with solutions having this range of concentrations. Then the proportions of the total amounts of ions originally present which are absorbed at equilibrium can be calculated from the equations given above. They are as follows:

Original external concentration.	Absorption.	
	Kation.	Anion.
20	0.12	0.07
4	0.23	0.03
1	0.50	0.01

If these numbers are compared with those actually obtained in experiments with manganese salts it will be seen that the calculated values for kation absorption are not violently discordant with those obtained experimentally. The calculated values for anion absorption are, on the contrary, not only in disagreement with those actually found, but are in the wrong order, for actually the proportion of anion absorbed decreases with increasing concentration.

Now Briggs and Petrie (1928) have pointed out that the observed absorption of both kation and anion to such an extent that the internal concentration of each exceeds that of the same ion in the external medium can be explained on the basis of ionic exchange if the protoplasm contains an immobile anion and the vacuole an immobile kation. Similarly, in such circumstances, the vacuole will absorb a larger proportion of anion from a weaker than from a stronger solution, and the observed influence of concentration on anion absorption might be explained on these lines. We may, therefore, at present conclude that the interchange of ions between tissue and external solution may be an important factor in the first phase of absorption. But until more data are obtained with regard to the absorption of a wider range of salts, and particularly of the distribution of electrolytes in the cell, the extent to which ionic exchange is responsible for the observed phenomena must remain problematic.

#### 4. *The later phase of absorption.*

As with potassium salts the earlier phase of rapid absorption is followed by long-continued absorption of both ions. In experiments with unchanged solutions of initially 0.001 M concentration or less, no equilibrium was reached by the end of four or five days and experiments with changed solutions also indicated that from 0.001 M solutions of manganese chloride absorption of both ions continued for at least twenty days. With 0.005 M solutions absorption generally became negligible after about five days, and the same appeared to be the case with stronger solutions.

The question arises whether the second phase of absorption is conditioned also by ionic interchange or whether, as we suggested for potassium salts, it might result from permeability changes in, or metabolic activity of, the tissue. As regards the first possibility, it may be that the first phase in absorption consists of a rapid exchange of ions between solution and the surface cells, and that this is followed by an exchange of ions between inner cells and the external solution, an exchange rendered slow on account of the low effective coefficient of diffusion of ions in the tissue. Permeability changes may be involved, and the fact that the second phase follows after a period of approximate equilibrium might be explained by supposing that the absorption of the manganese ion by the surface cells increases their permeability to ions, so that exchange with inner cells, at first impossible, is able to take place. The cessation of absorption from the more concentrated solutions after a time might then be explained as due to exhaustion of exchangeable ions in the tissue.

It has, however, been frequently suggested that divalent kations bring about

a decrease in permeability of protoplasm, and it might be argued that the rapid falling off in absorption at the end of the first phase is a consequence of this. Such a view would, however, leave the second phase of absorption unexplained.

It would also be possible to connect the second phase of absorption with respiration and explain the intake of manganese and the various anions in terms of an exchange of these with hydrogen and bicarbonate ions respectively (cf. Brooks, 1929; Briggs, 1930). The more rapid slowing down of absorption in the stronger solutions would then be expected if the higher concentrations of manganese affected the rate of respiration. In the absence of respiration data, however, we cannot come to any conclusion on this point, and in the present state of our knowledge we must leave it an open question as to how far the course of the second phase of absorption is conditioned by permeability changes, ionic interchange, or metabolic activity of one kind or another. Indeed, we should wish to defer any detailed discussion of the mechanism of salt absorption until our information is very much greater than at present.

#### SUMMARY

1. The absorption by carrot root tissue of both ions of manganese chloride, manganese sulphate, and manganese nitrate has been followed over periods of from four to eight days from solutions having initially concentrations ranging from 0.02 to 0.0002 M. The absorption was determined from the fall in concentration of the external solutions, the determinations of the ionic concentrations being made polarographically.

2. The absorption of all three salts followed much the same course from solutions of the same concentration. In each case the manganese intake showed a two-phase course, involving an initial phase of rapid absorption to a position of temporary equilibrium reached in about seven or eight hours, followed after a further twelve to twenty-four hours by the second phase of slow continuous absorption which was still proceeding from the weaker solutions at the end of the experiment. With stronger solutions absorption of manganese ceased or became negligible after three or four days. A two-phase course was not generally observable in the absorption of the anion, the rate of this absorption being at first much slower than that of the kation, but showing a tendency to exceed it later so that the disparity between the amounts of the two ions absorbed, at a maximum at the end of the initial phase of kation intake, tended subsequently to lessen.

3. The rate of absorption of both ions of each salt was affected by concentration, the rate of absorption relative to the concentration being less the higher the concentration. The effect of increasing concentration in reducing the rate of intake relative to the concentration was found to be much greater than in the case of potassium salts.

4. The relationship between concentration and total amount of manganese absorbed during the first phase is approximately given by the equation  $I = CE^k$ ,

where  $I$  and  $E$  are the final internal and external concentrations and  $C$  and  $k$  are constants. For all the salts examined  $k$  was found to have a value of about 0.3. This agrees with the value found by Laine for the absorption of manganese from manganese chloride by excised roots of *Phaseolus multiflorus*.

5. Although the relationship between concentration and intake is the same as that for adsorption, the further absorption of manganese which results when the external solutions are renewed is greater than would occur if the amount of absorption was solely conditioned by adsorption. The observed facts indicate that exchange of ions between external solution and tissue plays a large part in determining the amount of manganese absorbed during the first phase.

6. The factors determining the second phase of absorption are at present obscure. They may include ionic exchange between external solution and internal cells of the tissue, permeability changes, respiration, and other metabolic activities. Considerably more experimental data are required to enable a reasonably acceptable view of the mechanism of the second phase of the absorption of manganese salts to be put forward.

The work described in this paper was rendered possible by grants from the Research Committee of the University of Birmingham and the Government Grants Committee of the Royal Society. To both these bodies we would here record our thanks for their assistance.

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# Studies in the Physiology of Wood-destroying Fungi

## III. Progress of Decay under Natural and under Controlled Conditions

BY

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With five Figures in the Text

IT is obvious that decay of wood under natural conditions can proceed until the destruction of the material is complete, otherwise an accumulation of woody material would form on the floor of a forest. It is sometimes assumed that this destruction is the result of successive attack by a number of different organisms. If observations be made on the fungi growing on beech logs at various periods after felling, it will be found that there appears to be a sequence of species at various stages in decay. *Stereum purpureum* and certain moulds such as *Bispora* sp. and *Lasiosphaeria* sp. are usually the first fungi to be noticed, then *Stereum hirsutum*, *Polyporus adustus*, *Polystictus versicolor*, and *Trametes gibbosa* most commonly appear. Then the larvae of wood-boring beetles usually make their appearance and assist in disintegrating the wood. Finally Myxomycetes develop on the badly rotted wood. In a log of coniferous wood there is a similar succession, but different species of fungi are involved. Very soon after felling the sapwood becomes infected with so-called sap-staining fungi, e.g. species of *Ophiostoma*, *Hormonema*, *Cladosporium*, &c., then sap-rotting fungi such as *Polystictus abietinus* and *Peniophora gigantea* appear, while the greater part of the destruction of the heartwood is usually brought about by white species of *Polyporus*, such as *P. (Leptoporus) fragilis* and (on the Continent) *Lenzites sepiaria*.

How far the succession is accidental and dependent merely on the intensity of spore infection of the different species and the rapidity with which they develop, and how far it is a true succession conditioned by the slowly changing chemical composition of substratum are matters of interest. Is it merely a case of which fungus gets there first and occupies the territory? One may regard, mycologically, a freshly felled log of wood as freshly turned soil, open to chance seeding with any organism whose powers of dispersal happen to be the greatest. To give a precise answer in the case of any individual fungus would require special investigation, but we may draw certain general conclusions from the results of field observations and certain experimental work.

It is generally recognized that the fungi which decompose wood can be divided into two main groups:

- (1) Those which bring about a brown rot of the wood in which the cellulose and the associated carbohydrates are attacked but which leave the lignin more or less untouched.
- (2) Those which bring about a white rot in which all the constituents are depleted.

On theoretical grounds, therefore, it would appear likely that a fungus of the first type cannot bring about the total destruction of a piece of wood, while there should be no reason why one of the second type should not completely destroy the wood in which it is growing.

In the course of numerous tests made to determine the relative resistance to decay of various timbers a large number of figures have been obtained showing the loss in weight brought about by certain active wood-destroying fungi. Below are listed the maximum losses in weight which have been recorded for individual samples of certain perishable woods exposed to active wood-rotting fungi for a period of eight months at 22° C.

TABLE I

Timber.	Brown rot fungi.	Max. loss of dry wt. (%).	White rot fungi.	Max. loss of dry wt. (%).
<i>Abies balsamea</i> Mill.	<i>Merulius lacrymans</i>	59.8	—	—
" " "	<i>Coniophora cerebella</i>	58.9	—	—
<i>Acer pseudoplatanus</i> L.	<i>M. lacrymans</i>	64.0	<i>P. versicolor</i>	53.3
<i>Celtis</i> (?) <i>soyauxii</i> Engl.	<i>M. lacrymans</i>	61.5	<i>P. versicolor</i>	50.0
" " "	<i>C. cerebella</i>	62.0	<i>P. sanguineus</i>	61.0
<i>Ceratopetalum apetalum</i> D. Don.	<i>Lenzites trabea</i>	63.8	<i>P. versicolor</i>	58.9
" " "	—	—	<i>P. sanguineus</i>	41.2
<i>Picea sitchensis</i> Carr.	<i>Trametes serialis</i>	*66.5	—	—
<i>Virola merendonis</i> Pitt.	<i>M. lacrymans</i>	65.2	<i>P. versicolor</i>	63.7
" " "	<i>L. trabea</i>	58.7	<i>P. sanguineus</i>	74.6
<i>Vochysia hondurensis</i> Sprague	<i>M. lacrymans</i>	64.2	<i>P. sanguineus</i>	59.4
" " "	<i>L. trabea</i>	63.0	—	—

\* 31 weeks only.

It may be concluded from these figures that up to a certain stage of decay brown rot fungi can cause as great or even greater destruction of wood substance than white rot fungi, which are decomposing the more refractory lignin in addition to carbohydrates.

No individual loss in weight greater than 66.5 per cent. has ever in these tests been recorded in samples decayed by a brown rot.

It is of interest to note in this connexion that the cellulose content of most woods lies between 50 and 60 per cent., while the pentosans in coniferous woods amount to about 10 per cent., and in a hardwood such as beech may amount to 25 per cent. In a coniferous wood the lignin represents 25–30 per

cent. of the wood substance, while in a Dicotyledon grown in temperate climates it is usually round about 20–22 per cent.

If samples of wood are left exposed to a brown rotting fungus for considerably longer periods, little or no further apparent change takes place and the fungus slowly dies away, sometimes forming fructifications before it does so. If, on the other hand, samples are left in cultures of white rot fungi for longer periods, the fungus continues to grow. It was therefore decided to carry out a test in which the process of decay should be allowed to continue to its natural conclusion.

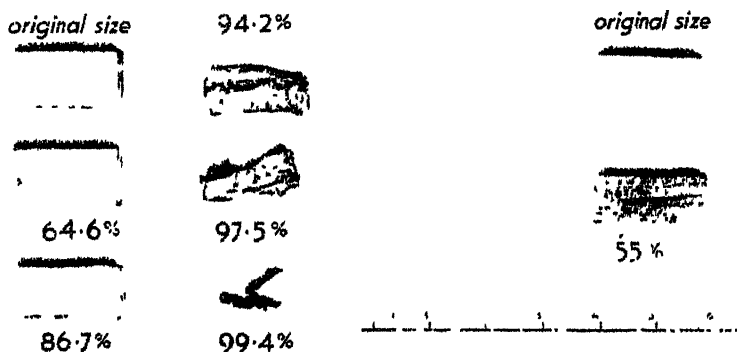
#### EXPERIMENTS ON PROGRESS OF DECAY UNDER CONTROLLED CONDITIONS

Samples of beech wood (*Fagus sylvatica* L.) measuring  $5 \times 2.5 \times 1.5$  cm. were oven-dried, weighed, and then, after being sterilized in an autoclave at

### Shrinkage due to Decay.

*Polystictus versicolor* (white rot)

*Phria vaillantii* (brown rot)



Beech

Scots pine

Figures represent loss in Dry Weight

FIG. 1. Photograph showing shrinkage of blocks of wood in various stages of decay.

15 lb. gauge pressure, exposed to fungal infection by being placed on cultures of *Polystictus versicolor* (L.) Fr. growing on 2 per cent. malt agar in special Kolle flasks. These flasks contain a reservoir for moisture in the neck, and sterilized water was run into this at intervals during the test so as to keep the atmosphere inside the flasks saturated.

The flasks, each containing four samples of wood, were kept at a constant temperature of 22° C. Twelve samples were removed at monthly intervals



until 9 months after infection, and thereafter at intervals of 3 months, till the last batch was removed 24 months after infection. The number of samples in a few of the later batches was less than twelve, as certain samples became contaminated and had to be rejected. After removal from the flasks the superficial mycelium was carefully removed from the samples, which were then weighed and, after oven-drying, re-weighed. In this way the final dry weight and the moisture content were determined. The final dry weight subtracted from the initial dry weight gives the loss due to the decay. In the last stages of decay it was difficult to remove all the mycelium, and in some of the samples after 24 months the mycelium must make up a large proportion of the final dry weight, for practically no wood recognizable as such remained. A photograph of blocks in various stages of decay is reproduced as Fig. 1. It will be noted that practically no shrinkage has occurred in a sample which had lost 64 per cent. of its weight as the result of a white rot, but that severe shrinkage took place in a sample that had lost 55 per cent. by the action of a brown rot.

TABLE II

*Progressive Loss in Weight of Beech Samples Exposed to Polystictus versicolor*

Period.	Average loss in dry weight (%).	Maximum loss in any one sample (%).	Period.	Average loss in dry weight (%).	Maximum loss in any one sample (%).
1 month . . .	7.4	10.3	8 months . . .	54.3	69.4
2 months . . .	19.7	23.0	9 „ . . .	67.6	77.5
3 „ . . .	26.2	33.4	12 „ . . .	75.7	83.1
4 „ . . .	31.7	37.4	15 „ . . .	77.9	80.0
5 „ . . .	37.6	47.5	(4 samples only)		
6 „ . . .	49.3	57.8	18 months . . .	85.2	87.9
7 „ . . .	55.5	67.1	(4 samples)		
			21 months . . .	90.5	97.1
			24 „ . . .	94.5	99.4
			(8 samples)		

These results are shown graphically in Figs. 2 and 3, from which it will be seen that the rate of loss in weight after a slight initial acceleration is more or less constant until about 75 per cent. of the original weight has gone, after which the rate of decomposition slows down. At this stage the blocks were covered with a thick, hard mycelial crust, and diffusion of the CO<sub>2</sub> respired by the fungal hyphae in the interior of the blocks may have been impeded. It has been shown by other workers that aeration of cultures on wood blocks accelerates their rate of decay. Though most wood-rotting fungi are resistant to a fairly high concentration of CO<sub>2</sub>, this factor is of greater importance when the fungus is growing in a large volume of wood in which diffusion of the gas is necessarily slower. There is no evidence from these or other experiments that anything in the nature of toxic 'staling' products are produced by

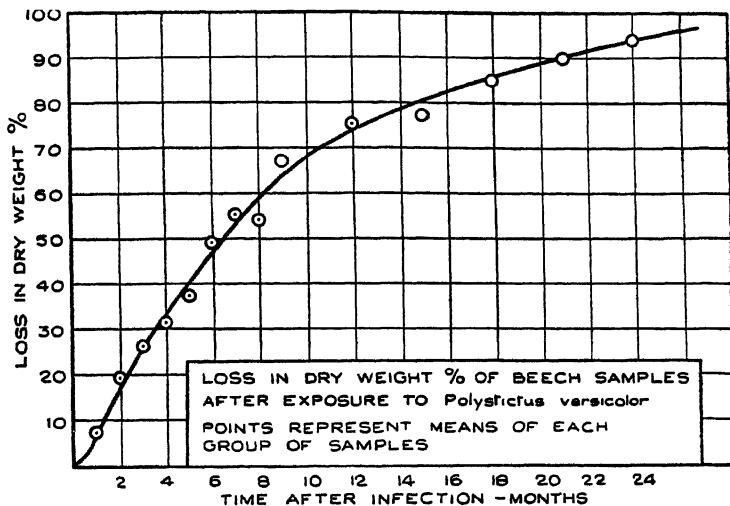


FIG. 2. Graph showing average loss in dry weight of groups of samples of beech exposed to action of *Polystictus versicolor* for various periods.

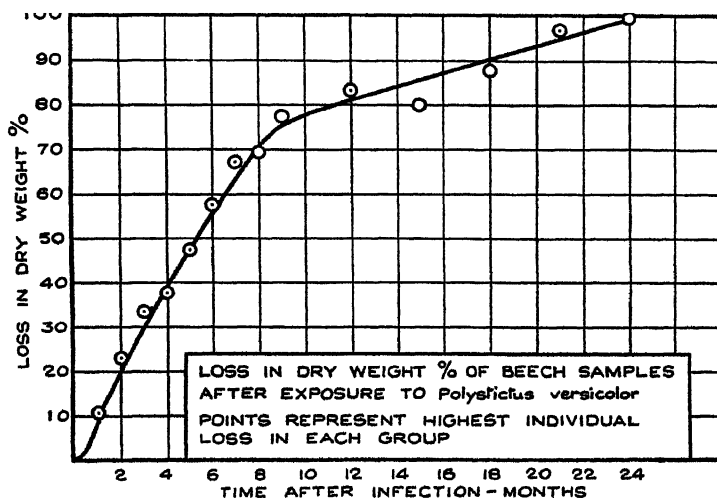


FIG. 3. Graph showing highest loss in dry weight of individual samples in groups of samples removed after various periods of exposure to *Polystictus versicolor*.

wood-rotting fungi. It is evident from the results of this experiment that the total destruction of a timber such as beech can be brought about by a single species of fungus which causes an active white rot, such as *Polystictus versicolor*, and that it is not necessary for the wood to be exposed to a series of fungi to bring about its complete disappearance.

In a similar experiment with blocks of Scots pine sapwood exposed to *Poria vaillantii* (D.C.) Fr., a fungus which causes a brown rot, less rapid

destruction of the wood occurred, and the maximum loss in weight recorded after twenty-four months was only 55.5 per cent.

#### MOISTURE CONTENT OF WOOD AT VARIOUS STAGES OF DECAY

The average moisture content of the beech samples removed after various periods is shown graphically in Fig. 4. It will be noticed that the moisture

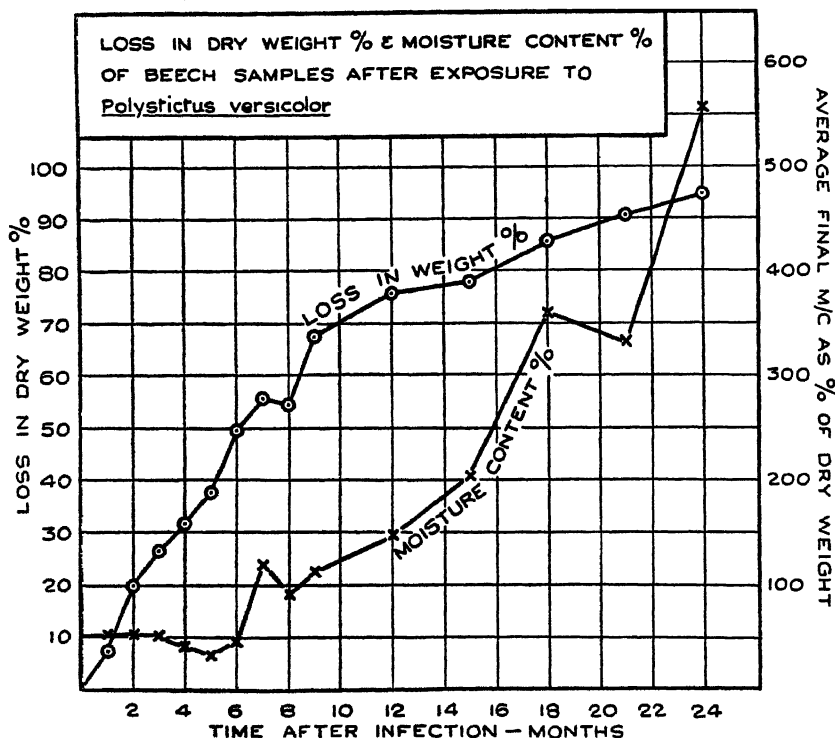


FIG. 4. Graph showing moisture content of beech wood samples after various periods of exposure to *Polystictus versicolor*.

content, which remained constant round about 50 per cent. (of the oven-dry weight) for six months, when the samples had lost about 50 per cent. of their weight, then began to rise, and that the rise roughly parallels the loss in weight. It should be emphasized that, while no exact control of moisture content was attempted, only sufficient water was added to the flasks to maintain the satisfactory growth of the fungus. It is evident that a much higher moisture content is required for growth in badly decayed wood than is necessary in sound wood, and that, after a certain stage in decay has been reached, the optimum moisture content for the growth of the fungus steadily rises as decay proceeds. Therefore it is possible only in the earlier stages of decay to speak of the optimum moisture content for any particular fungus in any timber. This change is no doubt related to the increased porosity of the wood in the later

stages of decay, which permits diffusion of air, in spite of the higher moisture content. In this connexion it may be noted that the optimum moisture content for fungal growth in sawdust (where there is, of course, good aeration) is round about 250 per cent. The very high moisture contents in the last three batches of samples are probably due to the presence in the wood of numerous fungal hyphae containing very little dry matter in proportion to their volume.

### SUCCESSIONAL DECAY

The effect of partial decay by a fungus causing a white rot upon the liability of the wood to decay by a fungus causing a brown rot and vice versa was investigated. The experimental method used was the same, except that somewhat larger blocks were used. In one set of experiments the blocks were sterilized after the first fungus had been allowed to act for four months, and in the second set the blocks containing living fungus were transferred aseptically to cultures of the second fungus and the two fungi allowed to act on the wood simultaneously.

TABLE III

*Loss in Dry Weight (per cent. Original Dry Weight) of Beech Blocks after Four Months' Exposure to Fungi at 22°C.*

The data figures represent the averages of 10 samples			
<i>Polystictus versicolor.</i>		<i>Coniophora cerebella.</i>	
Sound wood.	Wood first decayed by <i>C. cerebella</i> (26·8% loss) and then sterilized.	Sound wood.	Wood first decayed by <i>P. versicolor</i> (40·9% loss) and then sterilized.
40·9	50·6	26·8	35·5

In this test both fungi made more rapid growth on wood which was already partially decayed than they did on sound wood. Lehmann and Scheible (1924) similarly found that preliminary inoculation with *C. cerebella* increased the growth rate of decay of wood by other fungi, such as *Merulius lacrymans* and *Stereum purpureum*.

TABLE IV

*Loss in Dry Weight (per cent.) of Beech Samples after Eight Months*

<i>P. versicolor</i> alone.	<i>C. cerebella</i> alone.	<i>P. versicolor</i> , 4 months; sterilized; <i>C. cerebella</i> , 4 months.	<i>P. versicolor</i> , 4 months; <i>C. cerebella</i> (both alive).	<i>C. cerebella</i> , 4 months; sterilized; <i>P. versicolor</i> , 4 months.	<i>C. cerebella</i> , 4 months; <i>P. versicolor</i> (both alive).
50·6	(30·9)*	61·7	62·7	63·3	39·8

\* As the samples had become too dry this figure is too low.

In this experiment, when the first fungus was killed before inoculation with the second the total destruction brought about was about the same whether

the white rot or the brown rot fungus was allowed to act first, but a lower loss was recorded when the wood containing living *C. cerebella* was exposed to *P. versicolor* than when *C. cerebella* was put on to wood with living *P. versicolor*. *C. cerebella* is known to maintain very acid conditions in wood, and probably the pH of the wood was too low for the optimum growth of the *Polystictus*. Although it is quite common to find a number of fungi growing on the same log, it will usually be found that the different species are confined to separate areas, sometimes bounded by dark zones, and that it is rare for their mycelia to mingle intimately in exactly the same areas. In practice two wood-destroying fungi have very rarely been isolated from the same area in a sample of decayed wood.

A second experiment was carried out on the rate of decay by white and brown rot fungi of partially rotted wood. The samples of beech rotted to varying degrees, which were removed from cultures of *Polystictus versicolor* after various periods in the progressive decay experiment (*vide* Table I), were sorted into groups. The samples were selected so that in each group the average loss of the weight lay between two limits, i.e. between 15 and 20 per cent., 20 and 25 per cent., &c.

The samples were then sterilized and exposed for three months at 22°C. to cultures of *Coniophora cerebella*, and the loss in dry weight of each group was determined as before.

## RESULTS

TABLE V

Preliminary loss in weight (%) from action of <i>P. versicolor</i>	(Control)	0	Up to 15	15-20	20-25	25-30	30-35	35-40	40-45	45-50	50-55	55-60	60-65	65-70
Subsequent loss due to <i>C. cerebella</i>		23.1	16.1	21.3	23.2	21.7	27.5	24.5	29.7	27.4	35.5	28.4	37.4	33.2

These results indicate that until the wood has lost about 40 per cent. by the action of *P. versicolor* it is not significantly more readily decomposed by *C. cerebella*, but that beech wood severely decayed by *P. versicolor* is rotted more rapidly than sound wood. It may be noted in this connexion that the blocks tested in the first successional experiment had lost about 40 per cent. by the action of the white rot before they were exposed to *C. cerebella*.

A similar experiment reversing the order of attack by the fungi was then carried out. Sample blocks of Scots pine sapwood rotted to varying degrees by *Poria vaillantii* were sorted into groups according to their loss in weight, and then exposed for three months along with groups of sound blocks to attack by *Polystictus versicolor*. The results are given below and shown graphically in Fig. 5.

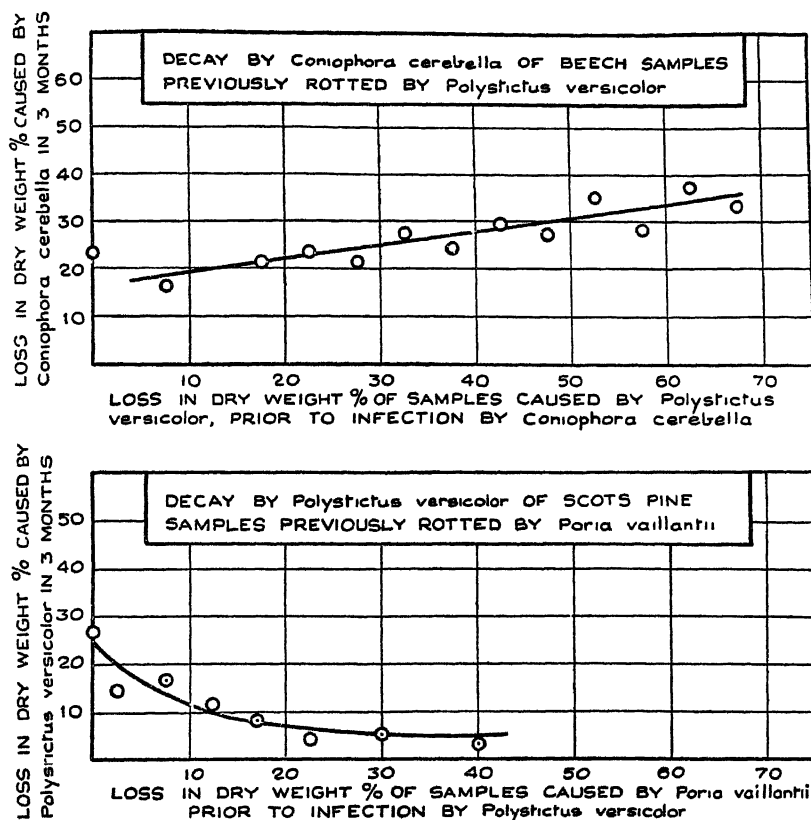


FIG. 5. Graph showing effect on subsequent rate of decay of wood samples of previous partial decay by a white rot and by a brown rot fungus.

TABLE VI

Preliminary loss (per cent.) in dry weight by <i>Poria</i> <i>vaillantii</i>	o (Con- trol)	Up to 5	5-10	10-15	15-20	20-25	25-35	35-45
Loss (per cent.) due to subse- quent exposure to <i>P. versicolor</i>	27.0	14.7	16.9	11.9	8.3	4.4	5.3	3.6

These results indicate that the removal by a brown rot of the cellulose in a coniferous wood renders it less suitable for subsequent attack by a white rot, such as *Polystictus versicolor*. It is unlikely that pH is concerned here, for the *P. vaillantii* was not alive when the blocks were exposed to the second fungus; also the pH would not appreciably change after the blocks had lost 10 per cent. of their weight, therefore some other factor such as depletion of cellulose must be responsible for the change in subsequent rate of decay.

*P. versicolor* does not normally attack coniferous wood, and it is evident that depletion of the cellulose and pentosans renders it much less suitable a medium for this fungus.

It is doubtful whether partial decay by a brown rot has any appreciable effect on the rate of decay of the wood by another fungus having the same chemical action (i.e. another brown rot). The rate of decay by *Paxillus panuoides* (a brown rot) of wood already partially rotted by another brown rotting fungus was found by Findlay (1932) not to be significantly different to the rate of decay of sound wood. It has been suggested that *Merulius lacrymans* grows more vigorously on wood which is partially rotted by *Coniophora cerebella*, but no conclusive evidence has been brought forward to prove that this is the case, and *Merulius* can undoubtedly grow very vigorously on wood which has not been previously attacked by another fungus. Possibly the acidity produced by *Coniophora* may stimulate the germination of the *Merulius* spores, but it is by no means essential for their development.

Groom (1927) suggested that incipient heart rot in the living tree may 'pave the way for subsequent fungoid attack'. The present author has carried out a large number of tests to determine whether incipient decay by heart-rotting fungi, such as *Fomes annosus*, does render the wood more susceptible to the attack of *Merulius lacrymans* and similar fungi. In some, but not in all the experiments, the partially rotted wood was more readily decayed by the dry rot fungi, and further work is required before a definite answer can be given.

## DISCUSSION

From the results of the experiments with *Polystictus versicolor* it is evident that the complete destruction of wood may be caused by a single species of fungus which brings about a white rot in which all the constituents of the wood are decomposed, but not by a fungus which causes a brown rot. This is confirmed by field observations: it is not uncommon to find the timber in the heart of a tree such as oak almost entirely replaced by fungal mycelium where a heart rot has been caused by a white rot fungus, such as *Polyporus dryadeus* or *Ganoderma* sp. Where, on the other hand, the decay has been caused by a brown rot, such as *Polyporus sulphureus*, a fair proportion of dark-coloured lignin-like material remains. Decay of the brown rot type cannot remove more than 65–70 per cent. of the wood substance, leaving a residue more or less entirely composed of lignin or lignin-like material which is resistant to decomposition by such fungi. While white rot fungi can destroy lignin along with cellulose, &c., they are unable to grow on a substratum consisting almost entirely of lignin. If, therefore, a brown rot proceeds as far as is possible, there remains a residue which will disintegrate only very slowly. Decay of woody materials by a brown rot will therefore lead to the formation of a much higher percentage of organic residues than decay by a white rot. White rot fungi, on the other hand, produce very much more mycelium in proportion

to the amount of wood which they decay than do brown rot fungi, and the ultimate fate of this mycelium, which in its turn may be decomposed, is practically unknown. The residual lignin complex from a brown rot will slowly be converted in the soil to humic materials. Since the most important decays of coniferous woods are brown rots, while on the other hand the commonest rots of hardwoods are white rots, it is evident that the contribution to the humus in the soil from the decomposition of the two types of wood is likely to be very different. A very great deal of attention has been devoted to the study of the humus derived from non-lignified or slightly lignified plant remains, such as leaves and straw, but much less is known about the role played by the residues from the decomposition of woody materials. This subject would seem to merit further consideration by soil chemists, for it is possible that the lignin-like residues may provide a useful source of humus. For the cultivation of various humus-loving plants, it has been found useful to embed stumps of wood in the soil forming a so-called 'rootery'.

Field observations and laboratory experiments suggest that only to a limited extent is it true to speak of a successional flora in the process of decomposition of a log of wood. The first organisms to appear are those which feed on the readily available food materials, such as sugars in the sap, and starch in the parenchyma cells of the sapwood, then true wood-rotting forms appear, and if the fungus is one which brings about a white rot, the complete disintegration may be caused by a single species acting alone. While a number of such species may colonize a single fallen log, they are usually limited to the area around the point of original infection. Where the log is attacked by brown rot fungi, approximately 30 per cent. of the material may be left; this crumbles away and becomes incorporated in the soil, where it undergoes slow decomposition by the micro-organisms of the soil.

If owing to some unfavourable influence, such as drought, the fungus growing first in the wood is killed off, other fungi can take up the process of decomposition, and it has been shown that preliminary decomposition, even to an advanced stage, by a white rot renders wood more readily rotted by a brown rot. Wood slightly decayed by a brown rot is rotted by a white rot as quickly or rather more quickly than sound wood, but when in an advanced stage, brown rot is not readily followed by a white rot.

## SUMMARY

1. The progressive loss in weight of wood decayed under controlled conditions by pure cultures of wood-rotting fungi was followed. It was found that the white rot caused by *Polystictus versicolor* can completely destroy samples of beech wood in two years. On the other hand, brown rots caused by fungi such as *Coniophora cerebella*, *Merulius lacrymans*, or *Lenzites trabea* which leave the lignin virtually unattacked, cannot remove more than about 70 per cent. of the wood substance.



2. Investigations have been carried out to determine the effect on the rate of decay of wood by brown and by white rot fungi, of exposing it first to preliminary rotting by a fungus causing a different type of decay. It was found that preliminary rotting of beech with a white rot increases the subsequent rate of decay by a brown rot fungus.

3. The significance of these results is discussed in relation to the decay of fallen timber and the supply of humus to a forest soil.

The work described above was carried out as part of the programme of the Forest Products Research Board and is published by permission of the Department of Scientific and Industrial Research.

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# Caytonia

BY

TOM M. HARRIS

(*University of Reading*)

With Plate VII, and four Figures in the Text

## INTRODUCTION

THIS paper is an account of the Caytonialean fruits collected in 1938 by Mr. F. M. Wonnacott and deposited in the Geology Department of the British Museum. It follows that of the Caytonialean leaves in the Wonnacott collection (Harris, 1940), and it is hoped that it will eventually be possible to contribute a paper on the pollen-bearing organs. The fossils were obtained on the shore of Cayton and Gristhorpe Bays from the famous plant bed which is of Middle Estuarine (Bajocian) age.

Thomas (1925) established the Caytoniales in his well-known paper. The great interest of the family and advances in technique justify a re-examination of Thomas' findings; moreover, Thomas himself made it clear that far less is known about the Caytoniales than might be expected from these favourably preserved compression fossils.

The main new points now contributed are: (1) The pollination of the two Yorkshire species was gymnospermous; the structure admitting the pollen is described. (2) The genus *Gristhorpia* is shown to be so like *Caytonia* that they are united. (3) The foliage of the two fruit species is related to them.

The material consists of a few hand-specimens in a general collection and a large number obtained by bulk-maceration of some blocks of the basal shale. For this maceration dilute HCl followed by alkali served; this yielded the fossils in an unaltered state.

## TERMINOLOGY

Although most of Thomas' terms have been used a few changes have been made.

The central part of the megasporophyll (or 'infructescence') is termed the rachis instead of the 'axis' as this might suggest a stem. The term 'carpel' for the individual fruits has been dropped (as had been done earlier by Edwards in 1929), this term having theoretical rather than descriptive implications.

The part termed the stigma by Thomas is not stigmatic; it is described here as the lip of the fruit and the gap between this and the stalk is described as the mouth of the fruit.

Some emendation of the diagnosis of *Caytonia* and of the two species is made necessary by the present work.

*Genus Caytonia; emended diagnosis.*

Megasporophyll dorsiventral bearing fruits laterally in subopposite pairs. Fruit shortly stalked, its wall forming a sac with a lip situated close to the stalk, and a narrow mouth between the stalk and lip communicating with the interior. Seeds small, orthotropous, their micropyles facing the mouth of the fruit; borne in a curved row on the fruit wall. Integument single, nucellus free to the base. Pollination by pollen reaching the micropyle.

This genus, the leaf *Sagenopteris*, and the microsporophyll *Caytonanthus* constitute the *Caytoniales*.

*Caytonia sewardi* Thomas.

1925 *Caytonia sewardi* Thomas, p. 315, pl. xii, figs. 14-24; xiii, figs. 25-32; xv, fig. 48.

1933 *Caytonia sewardi* Thomas: Harris, p. 111 (comparison with *C. thomasi*).

1934 *Caytonia sewardi* Thomas: Thomas, p. 193, text-fig. 15. (Details of fruit.)

*Emended diagnosis.* Fruits spherical containing about eight or fewer seeds; epidermal cells of fruit showing jagged thickenings, mouth of fruit small, lip curved round the stalk; cuticle of mouth showing about eight thick bars. Testa of seed thick, its surface conspicuously marked with isodiametric pits.

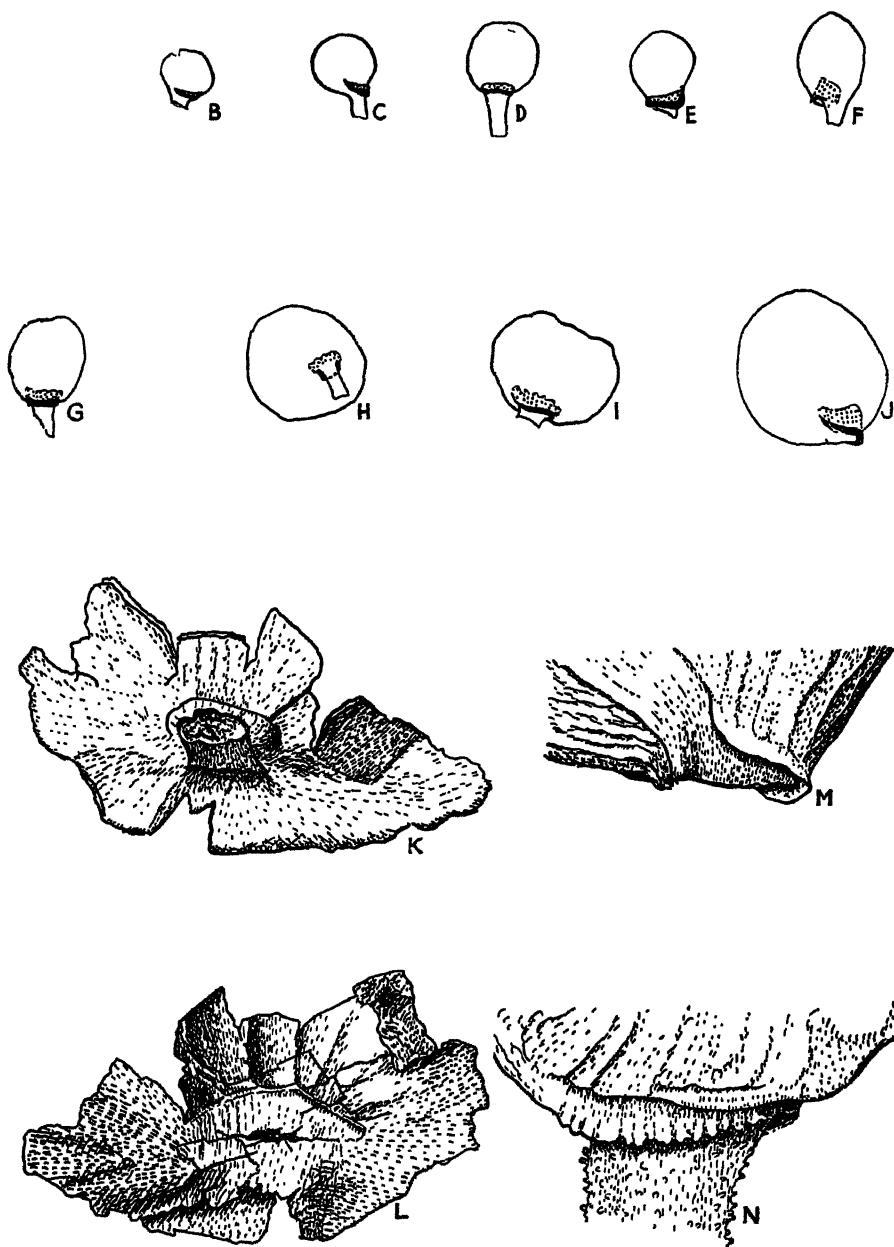
## DESCRIPTION

The material consists of a large number of isolated fruits and seeds.

The largest fruits are 4.5 mm. wide, most are about 3.5 mm. wide, while the smallest found was less than 1 mm. wide. Large fruits yield about eight seeds (eight and a small abortive one in the largest examined), smaller ones as a rule yield a smaller number of well-developed seeds. Certain small fruits are, however, of arrested development, and contain imperfectly developed seeds. These specimens throw light on the stages of growth of the normal fruit (p. 730).

The seeds are arranged in the compressed fruit in a way fully consistent with Thomas' conclusion, though in view of what is now known for the other species they are perhaps better described as forming a curved row than as two rows.

The fruit stalk, which is short and slender in this species, shows dorsiventral cuticle structure; the cuticle of the side regarded as adaxial or upper (i.e. the one with the mouth of the fruit) shows rows of epidermal cells with thick lateral and thin end walls; the lower side shows cells in less obvious rows; longitudinal walls and end walls are of about equal thickness and both show



TEXT-FIG. 1. *Caytonia seawardi*. A-G is a series of small (immature) fruits without seeds; H-J are normal fruits. The projecting lip is shown in black, the cuticle of the mouth is stippled, all  $\times 5$ . K, L, ventral wall of fruit, K from the outside showing the lip and stalk, L from the inside showing a ridge opposite the mouth from which shallow grooves pass upwards; the cracks were caused in preparation,  $\times 20$ . M, details (by reflected light) of the fruit shown in J,  $\times 20$ . N, details by reflected light of the lip of the fruit shown in J,  $\times 40$ . The collection numbers are as follows: A, V. 26637; B, V. 26638; C, V. 26639; D, V. 26640; E, V. 26641; F, V. 26642; G, V. 26643; H, V. 26644; I, V. 26645; J, V. 26646; K, V. 26647.

jagged projections. Unicellular hairs are common on the upper, rare on the lower side.

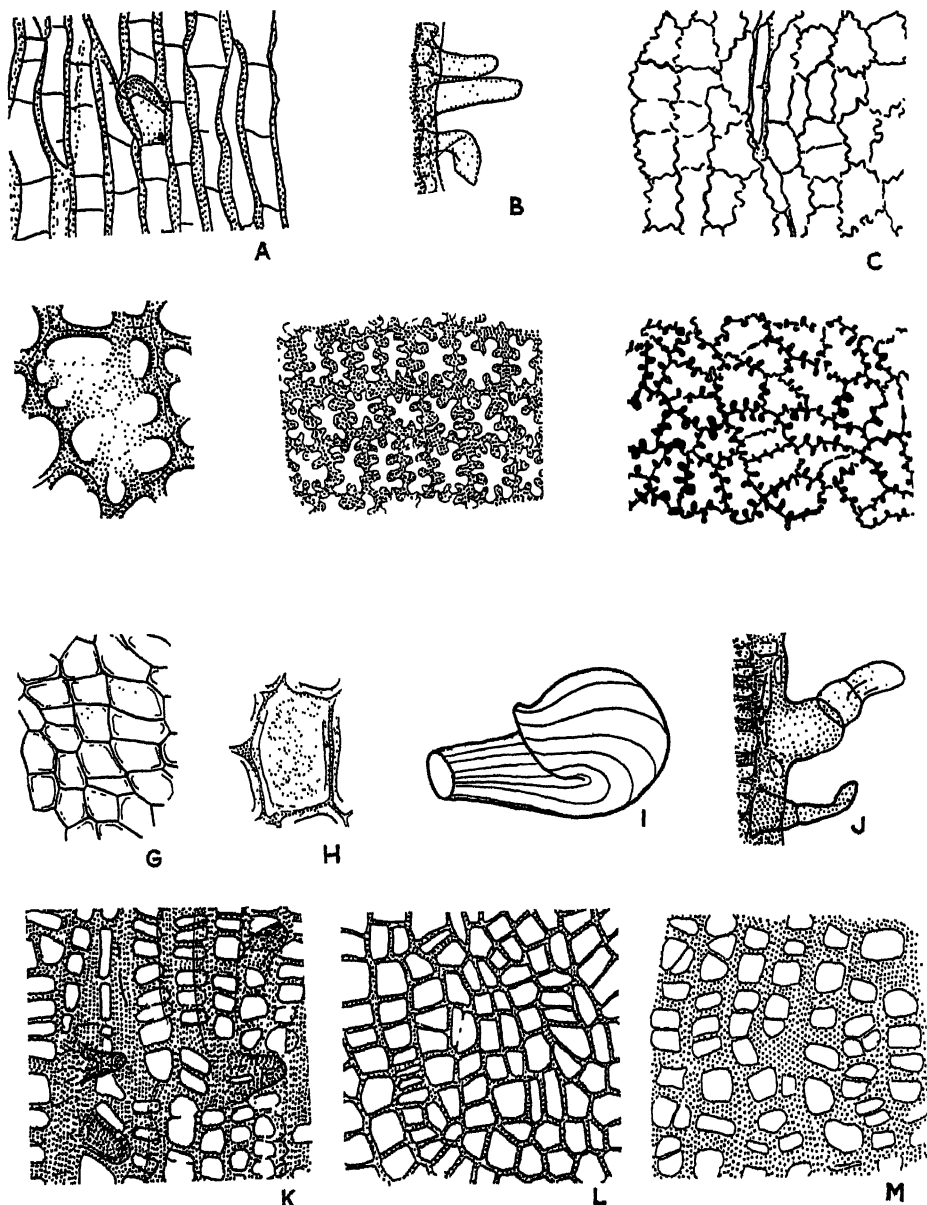
The fruit has a thick cuticle ( $10\ \mu$  thick); the cells are roughly isodiametric and form obscurely marked longitudinal rows except near the lip, where they are narrow and in obvious rows. The cell rows pass round the back or sides of the fruit to end on the lip just as in *C. nathorsti*, but owing to the smaller size of the lip they converge more. The cell-walls are fundamentally straight but are provided with strongly marked jagged thickenings which give a suggestion of sinuosity. The surface of the cells is obscurely mottled. No hairs or stomates were seen.

The lip (stigma of Thomas) is a ridge forming a semicircle on the upper side of the stalk. Its width (measured round the curve) is  $800\ \mu$ – $1200\ \mu$ ; it only extends about  $150\ \mu$  outwards from the fruit wall in the present specimens (Text-fig. 1, K, M, N), though in the specimen figured by Thomas it extended some distance along the stalk. The lip is rather thickly cutinized and is composed of small cells with a bulging surface, but the papilla development is less pronounced than in *C. nathorsti*.

Most fruits when macerated yield traces of the tissue of the flesh and in some it is well preserved. This tissue consists of a few layers of large round or oval cells separated by large spaces. The substance of the walls is fairly resistant to maceration, but less so than are the cuticles of the fruit and seed; when macerated it also stains more strongly. It thus resembles the cell casts of the fibrous layer of the seeds and of the mesophyll cells of the leaf *Sagenopteris colpodes* (Harris, 1940), and the same explanation of its preservation is put forward, that the cell contents sometimes remained as an oily layer lining the wall and became converted to hard resin. Cells of this flesh are visible on the inside of fruits which had been broken before preservation and can also be demonstrated by grinding away the side of a fruit and etching the surface with warm  $\text{HNO}_3 + \text{H}_2\text{CrO}_4$  followed by alkali. In one of these etched sections a group of narrow cells lies in the inner part of the flesh, but no tracheid thickenings were recognized; the vascular structure of the fruit is thus still unknown.

In a fruit which had been broken and filled with matrix, the upper (lip) side showed a number of ridges which pass from the mouth over the inner surface towards the seeds, though their exact relation to the seeds was not followed. These ridges correspond to those seen in the cuticle of the mouth, and it seems likely that the discovery of further specimens of this sort might give valuable information.

Small and barren fruits are rather common; a representative series is shown in Text-fig. 1 A–G, and a similar series is known for *C. nathorsti*. Most of these fruits have fairly thick cuticles, and although their epidermal cells are fewer and smaller than those of normal fruits, it is considered unlikely that such thick cuticles would be able to stretch enough to allow for the necessary growth. Most probably their normal development was arrested at some stage,



TEXT-FIG. 2. A-F, *Caytonia seawardii*. A, upper side of fruit-stalk (V. 26648),  $\times 250$ . B, hairs of fruit-stalk (V. 26640),  $\times 250$ . C, lower side of fruit-stalk (V. 26648),  $\times 250$ . D, cell of fruit-wall (V. 26649),  $\times 800$ . E, heavily thickened fruit-wall (V. 26650),  $\times 250$ . F, lightly thickened fruit-wall (V. 26649),  $\times 250$ .

G-M, *Caytonia nathorstii*. G, cells in region of lenticel (V. 26658),  $\times 250$ . H, details of a cell from a lenticel (V. 26658),  $\times 800$ . I, diagram of a small fruit to indicate the direction of the epidermal cell-rows. J, hairs on stalk (V. 26659),  $\times 250$ . K, upper side of fruit-stalk (V. 26660),  $\times 250$ . L, lower side of fruit-stalk (V. 26660),  $\times 250$ . M, fruit-wall (same specimen as G),  $\times 250$ .

but the cells remaining alive, the cuticle continued to be formed, so that although they may not be strictly young stages, they may reflect them rather closely and thus show how the fruit developed.

The smallest fruits of *C. sewardi* and *C. nathorsti* are almost identically similar except for their epidermal cells, the lip is about the same width as the stalk, and the sac is only slightly wider. In the larger fruits the stalk and lip have increased in size a little, but the sac very considerably, and in normal mature fruits of *C. sewardi* it is about ten times wider than the stalk. In *C. nathorsti* the development is the same except that the growth of the sac in relation to the stalk and lip is not so disproportionately great. In these arrested fruits there is a cuticle lining the mouth, but its structure is slightly different (p. 724). No specimen was found in which the mouth was actually open. It is unknown at what stage pollination occurred; it happens that the fruits which yielded pollen in their seeds were all mature, but no conclusion must be drawn from any failure to obtain them from immature fruits.

The structure of the mouth of the fruit is described and discussed on p. 721. The seed is discussed on p. 724.

*Caytonia nathorsti* (Thomas) n. comb.

1920 *Laconiella sardinica* Krasser (inadequate description with no figure).

1922 *Gristhorpia* Thomas, p. 452 (*nomen nudum*).

1925 *Gristhorpia nathorsti* Thomas, p. 305, pl. xi, figs. 1-13; xiv, figs. 41, 42; figs. 43-47 (full description and figures).

1992 *Gristhorpia nathorsti* Thomas: Edwards, p. 386; pl. iv, figs. 1, 2. (description and figures).

1933 *Gristhorpia nathorsti* Thomas: Harris, p. 111 (comparison with other species of *Caytonia*).

The only available specimen of a megasporophyll, V. 26661, was made into a balsam transfer. It still bears five fruits and shows scars of eleven others.

The rachis shows well-marked dorsiventral structure; on one side (regarded as lower) it bears a number of longitudinal ridges, on the other it shows oval scars where fruits have fallen off. This arrangement of the fruits had already been inferred by Thomas from other evidence.

The ridges are of interest. On the upper side there are only two which run from scar to scar; on the lower side there is a broad ridge along the middle and a smaller one just inside each margin; these ridges run uninterruptedly, but in the lower part their number appears to be increased by supplementary ridges. Thomas (1925, p. 305) mentioned such ridges but supposed that they were caused in compression; Walton's (1936) work, however, has shown that it is unlikely that compression should cause this effect; more probably the ridges are original features due to ribs of mechanical tissue.

The cuticle of this specimen agrees with Thomas' account, though the dorsiventral structure described by him is feebly shown; the cells are, how-

ever, appreciably shorter and form better marked rows on the upper side. Both sides bear a few small unicellular hairs of conical shape.

The oval scars were examined closely in the hope they might reveal vascular bundle prints, but none was recognized; they have no cuticle or other resistant layer.

The fruit-stalk or pedicel was studied in many specimens of detached fruits (Text-fig. 2, J, K, L). The cuticle was only briefly mentioned by Thomas; it is of dorsiventral structure. The upper side shows rows of short cells with grossly thickened lateral walls. Many unicellular hairs occur on this side and on the margins; in some specimens hairs of two or three cells also occur. The lower side shows less regularly arranged cells with more evenly thickened walls, and as a rule no hairs occur here.

As the megasporophyll is detached its orientation is unknown, but two pieces of evidence suggest that the side on which the lips of the fruits occur faced upwards.

(1) As Thomas showed, the two sides of the megasporophyll rachis are different and correspond closely to the upper and lower sides of the petiole. This I have confirmed with the present material.

(2) The ridges on the megasporophyll rachis are arranged like those on many Dicotyledon leaf midribs where the adaxial side shows two strong ridges running from the base of one leaflet to the next, the abaxial side several ridges. Leaflets too are normally borne above the middle line of a midrib or rachis.

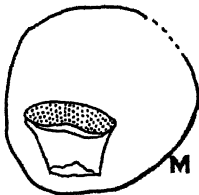
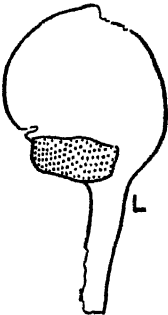
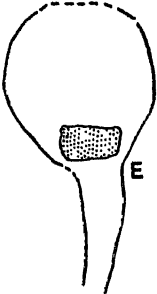
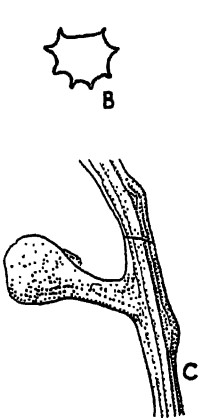
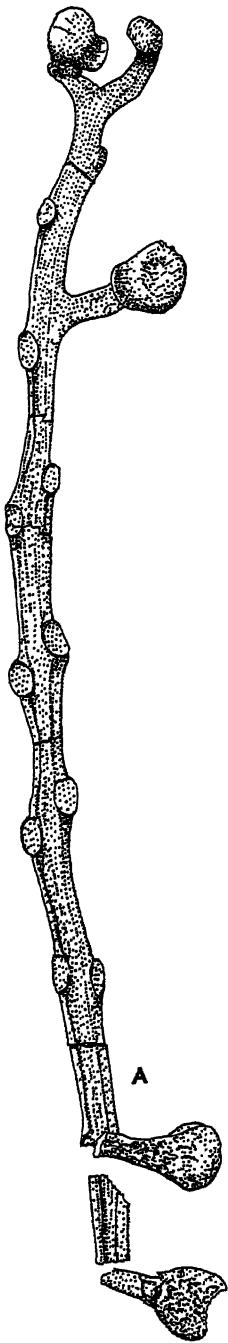
The normal fruit is more or less spherical, but as in *C. seawardi* there is a series of small barren fruits which are probably of arrested development. A few fruits yielded fairly well-preserved seeds; that shown in Text-fig. 3 H shows nine seeds arranged in a curved group; in addition there were three seeds which were accidentally removed in preparation. Other fruits gave the following numbers 8, 9, 9, 9, 11, 12, 15, the probability being that a few were broken in preparation and so missed in each case.

The arrangement of the seeds was not hitherto known; it appears that the arrangement is the same as in *C. seawardi* and *C. thomasi*, the number being intermediate.

The fruit has a thick cuticle which shows the outlines of straight-walled cells which are arranged in longitudinal rows. The walls of the cells are grossly thickened and may obscure the outline of the cell, the thickness of the walls in the specimen shown in Text-fig. 2 M being by no means extreme. Often cells are arranged in small packets as though formed by division after the walls had become rigid. In the small fruits the cells are rather smaller and the lateral walls are not so thick.

In nearly all fruit cuticles two sorts of thin place occur. Some are oval regions  $40\ \mu \times 25\ \mu$  and almost certainly represent poorly preserved stomates; the others consist of a patch of 10–15 epidermal cells with abnormally thin cuticles. Such thin patches are often raised and broken in the middle, while





underneath them there extends a layer of ill-preserved but somewhat cutinized cells. Such thin regions strongly suggest lenticels; the cuticle of an apple fruit shows just similar thin patches over the lenticels, caused no doubt by the early isolation of these epidermal cells. These thin areas are of interest also in providing a picture of the probable state of the cuticle before extreme thickening occurred, and here the cells show exactly the same fine characteristics of a thick border round the middle lamella and a central sculptured area as do the cells of the upper epidermis of the leaf of *Sagenopteris phillipsi* (*sensu stricto*); see Harris, 1940.

The orientation of the cell rows of the fruit is noteworthy. In specimens where cell rows are easily made out, they run from the lower side of the stalk, over the back and down across the front of the fruit to the lip. Other rows nearer the margin make a shorter course, curving round again to converge on the lip. This points to the lip being the true apex of the fruit (Text-fig. 2 I.)

The form of the lip of the fruit in these specimens agrees with those described and figured by Thomas (1925). I did not succeed in finding any pollen on the lip, but my methods (preliminary bulk maceration) may have removed them, and in any case this is no longer considered important.

#### *The mouth of the fruit of Caytonia.*

The small gap between the fruit-stalk and lip narrows and ends at a short distance above the lip, but the cuticle of the fruit-stalk and lip continue upwards in conjunction with one another for a considerable distance. This region, which is a flattened tube, is called the mouth. Its essential structure appears to be the same in the three species, but there are differences between them of dimensions.

It is easy enough to dissect out the cuticle of the mouth from a macerated fruit, but owing perhaps to its thickness and the effects of compression it has proved most difficult to understand its features, and this account while adding somewhat to what was known from the work of Thomas (1925) and of Harris (1933) still leaves much in doubt.

The cuticle of the mouth in *C. sewardi* is a band 1 mm. long, nearly 0.5 mm. wide, curved in a semicircle round the stalk; the lower margin forms the lip, the upper projects into the fruit. That of *C. nathorsti* is twice as large (2 mm.  $\times$  1 mm.) and less curved; that of *C. thomasi* is longer (2.6 mm.  $\times$  0.7 mm.)

TEXT-FIG. 3. *Caytonia nathorsti*. A, transfer of megasporophyll showing fruit-scars (the two lower fruits are displaced) (V. 26661),  $\times 5$ . B, restoration of transverse section through the rachis. C, part of A before transfer showing the ridges on the lower side. D-J, L, M, series of fruits at various stages of development, all  $\times 5$ . The cuticle of the lip and mouth are stippled. In H nine seeds are seen in position, three others were removed in preparation. K, outline of megasporophyll before transfer,  $\times 2$ . D, V. 26662; E, V. 26658; F, V. 26663; G, V. 26659; H, V. 26664; I, V. 26665; J, V. 26666; L, V. 26667.

and still less curved. The band of cuticle is divided into a number of more or less distinct zones which are:

- (1) The open part of the mouth where the cuticles of lip and stalk have not met.
- (2) An intensely thick middle zone.
- (3) A thinner zone within the fruit forming the upper margin of this band.

In addition to these zones which run from end to end of the band there are indications of structure running across it. At the lip are obscurely thickened regions which passing up into zone 2 become distinct while in zone 3 are very obvious indeed; for these the descriptive term 'transverse bars' is used.

The widths of zones 1-3 in *C. sewardi* are about 0.15, 0.15, 0.2 mm. respectively; in *C. nathorsti* zone 1 is very much broader, being about 0.7 mm. wide, though over much of this partial union of the two layers of cuticle occurs; zone 2 is about 1.0, zone 3 about 0.2 mm. wide. In *C. thomasi* the widths of the three zones are each about 0.23 mm.

Some difference is also to be seen between the transverse bars. In *C. sewardi* where they are the plainest they are not parallel but diverge as they enter the fruit; in the other two they are almost parallel. Their number is 6-9, most often 8 in *C. sewardi*; 12-20 in *C. nathorsti*; and about 30 in *C. thomasi*. The agreement between these numbers and the maximum number of seeds in the fruits of each species is stressed later.

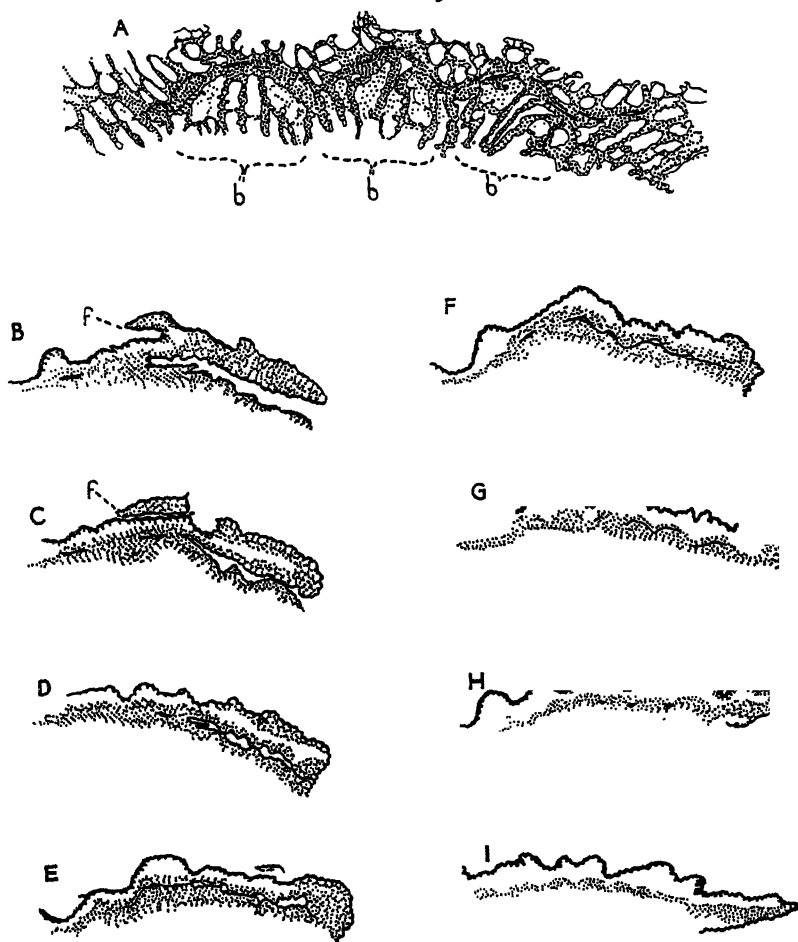
The three zones will now be described in detail.

Zone 1 is narrow and not easily distinguished in *C. sewardi* owing to the great thickness of the cuticles, but apart from their greater thickness the cells agree with those of the adjacent regions the stalk and lip. Sections through the cuticles of fully macerated fruits showed that the union of the two cuticles takes place along a zigzag rather than a straight line so that the cavity of the mouth has a number of small upward extensions.

In *C. nathorsti* zone 1 is far better developed and easier to investigate. This zone is sometimes smooth as in the specimen shown in Pl. VII, Fig. 5, but often shows well-marked transverse bars which are the continuation of those of the next two zones (Pl. VII, Fig. 3). The bars are dark because their cuticle is about twice as thick as it is between; for the same reason the surface of the cuticle of the mouth can be seen to be corrugated from whichever side it is examined.

Sections through this region of the mouth show the two layers of cuticle fairly distinctly; there is sometimes a minute crack between them, but no spaces of any appreciable width.

Zone 2, the heavily thickened region, is best developed in *C. sewardi*, where the thickness of the double cuticle is as much as 60  $\mu$ . This zone is rather conspicuously marked by the transverse bars which owe their appearance partly to inequality of thickness, partly to variation of inclination of the cell-walls. The great thickness of the cuticle is due largely to the extension of cutinization down the anticlinal walls, this being especially true of the stalk side, where the cells are actually of columnar shape. Owing to the effect of compression on



TEXT-FIG. 4. *C. seawardi*. Sections from a transverse series of the cuticles of the base of the fruit cut from below upwards at  $8\ \mu$  thickness (V. 26651) 'A', part of the mouth from the 35th section. The crack between the two layers of cuticle has become obscure, but the bars of thickening (*b*) of the lower part are clear;  $\times 250$ . B-I, semi-diagrammatic drawings of sections in which the outer or periclinal wall of cuticle is shown in black but masses of anticlinal walls are merely stippled, all  $\times 50$ . In this series the lip is first seen in section 12 (not shown); in B (section 23) the mouth is open, in C (section 25) it is closing, in D (section 27) it is closed apart from small canals which become smaller in E (section 29) and F (section 31). In G (section 35) the canals are obliterated and the crack between the two layers is hard to trace (see above). In H (section 43) the cuticle of the mouth is becoming thinner, in I (section 49) it is still thinner, and in section 52 (not shown) it is last seen.

The lip and ventral side face upwards, the dorsal side of the fruit wall was dissected away before cutting. In B and C there is a fold formed from the bent up margin of the lip (*f*).

such cells which do not stand vertically, a confused picture of many superimposed walls is seen, and even in section several cell-layers may appear to be present; but comparison of adjacent sections has convinced me that only the two layers occur—the almost cubical cells of the lip, side and the columnar

ones of the stalk side. In this zone also a distinct crack between the two layers is sometimes to be seen in sections.

Zone 3 begins with the thick zone but becomes thinner and then very thin indeed. Its structure is most easily observed in *C. seawardi*, where the transverse bars of thick and thin cuticle are most obvious. Surface observations show a herring-bone pattern in the thick bars formed by lateral walls of long cells which run from the thin sides obliquely inwards to the thick centre; these long cells are again the ones on the stalk side. On the lip side and between the thick bars the cells are isodiametric or slightly elongated.

The inner edge of this zone invariably shows an irregular edge as though it had been torn in manipulation, which is probably the case. Often the tear follows the thicker bars to produce a series of teeth (Pl. VII, Fig. 9) but this effect is certainly artificial. At some point above the torn edge the two epidermises may have separated to line the front and back of the fruit, but if the hypothesis given on p. 729 is correct, the fruit was so fleshy that no wide separation occurred till the seeds were reached.

In *C. nathorsti* the thickened bars in this zone are less pronounced and less extensive, so that towards the torn edge the cuticle is uniformly thin, though the bars remain recognizable by the slightly greater length of their cells.

In the small seedless fruits the cuticle of the mouth is often of nearly the same size as in the normal mature fruits, and even when rather smaller is disproportionately large in relation to the sac. Even in the smallest specimens it shows distinct transverse bars the number of which is typical for the species, and the structure is in fact very like that of the mature fruit except that the middle thick zone is either totally undeveloped, or in moderate sized specimens feebly developed (Pl. VII, Fig. 3). If it is correctly assumed that these small fruits are arrested at early stages of development it would follow that the mouth acquired its full normal structure, except for the thickening of the middle zone, before the seeds grew to their normal size.

#### *Seeds of C. seawardi and of C. nathorsti.*

The seeds of both Yorkshire species were described by Thomas in 1925. They were re-examined when another species, *C. thomasi*, which was particularly favourable for seed study, was described and certain differences of interpretation were reached. Dr. Thomas was good enough to discuss this work and expressed concurrence, but no new description has yet been given. It happens that the two species are so similar that they can be described together.

The seeds of *C. seawardi* can be obtained intact out of fruits, but the best specimens for study are isolated. The seeds of *C. nathorsti* have only been isolated from fruits in a state modified by chemical treatment, but these and specimens found by Thomas at the side of burst fruits serve to identify the isolated seeds.

The seeds are oval, those of *C. seawardi* about  $1.5 \times 1.0$  mm., those of *C. nathorsti* often a little larger, but both vary in size a good deal. A few speci-

mens show various distorted shapes which can be explained on the hypothesis that they were originally somewhat flattened and, while normally preserved with a flat side down, were occasionally preserved on edge or on end with consequent difference of outline on compression. The evidence suggests that in *C. seawardi* the breadth was about twice the thickness. The compressed substance of *C. seawardi* is about twice as thick as that of *C. nathorsti* and it is in consequence less apt to show distortion through inequalities in the matrix. In *C. seawardi* the substance measured 0.1–0.15 mm. in *C. nathorsti* about 0.05 mm. (Thomas' figure of 0.3–0.5 mm. for *C. nathorsti* is evidently a misprint). Both are black and shining, but while *C. nathorsti* is smooth, *C. seawardi* is covered with honeycomb-like pits. These pits represent the sunken interiors of the cells of the epidermis of the testa ('blow off layer' of Thomas). In *C. nathorsti*, where the outlines of the epidermal cells are harder to see, the cells are about twice as long as broad. In both the cells lie in longitudinal rows.

The seeds are composed of the following parts:

1. Integument (or testa), which has an inner and an outer cuticle enclosing the stone layer which forms nearly the whole thickness of the compressed seed. The micropylar canal, which is also cutinized, is at the apex and the hilum at the base.
2. A nucellus which is free and cutinized to its base. The chalaza is just opposite the hilum.
3. A megaspore membrane enclosed in the nucellus.

There is no evidence that the seed-coats were vascular, though elongated cells like tracheids can be seen in the funicle of *C. seawardi*. No trace of an embryo has been seen, and the absence of any appreciable carbonaceous matter in the megaspore shows that it contained no substantial tissue at the time of preservation.

The integument is covered by a rather delicate cuticle in which cell outlines are particularly difficult to see owing to lack of cutinization along the anticlinal walls. Where cell outlines can be seen, however, they agree with what would be expected from the surface of the seed, being elongated in *C. nathorsti*, isodiametric in *C. seawardi*, while the surface of the cell is flat in *C. nathorsti*, sunken in *C. seawardi*.

Thomas was able to show from his sections that this cuticle belongs not to the outer or 'palisade' layer of the stone, but a distinct cell layer outside it which he named the 'blow off layer'. He reached the conclusion that (in *C. seawardi*) the cells are cutinized on their inner walls, not their outer walls, as is ordinarily the case. This has not, however, been confirmed; it is considered that the appearance in section on which this view was based was due to the deeply sunken position of the whole outer wall into the middle of the cell.

At the apex of the seed there are two tracts where the cells are different; here cutinization extends inwards along the anticlinal walls, making the outlines of the cells very obvious. These two tracts lie along the sides of the

seed, that is in the plane in which it is normally compressed. They are obvious in *C. sewardi*, less so in *C. nathorsti*. They appear to form the basis in Thomas' restoration for the round cells forming a sort of inner integument emerging through the micropyle of *C. sewardi*. The cuticle of these cells is, however, continuous with that of the rest of the integument and there is a transition from the one to the other type of cell, so that the simpler explanation that they are part of the epidermis of the integument is adopted.

At the chalaza this cuticle shows no characteristic feature, being merely torn.

The structure of the interior of the integument was elucidated for *C. sewardi* by Thomas (1925) by means of thin sections. He showed that the substance consists of two layers of thick-walled cells, an outer of short erect prisms forming the 'palisade' layer, an inner of longitudinally placed pointed cells the 'fibrous' layer. This structure was seen also in *C. thomasi* by using Thomas' methods (Harris, 1933) and has been confirmed for *C. sewardi* by using the method of polishing a surface and then etching. In *C. nathorsti* there is as yet no information about the cells of the testa.

A peculiarity of *C. sewardi* is that the lumen of these cells is often filled with a material giving an internal cast. That of the 'palisade' cells consists of a small cylindrical mass from which fine processes emerge, these representing the canal-like pits of the stone cell. That of the 'fibrous' layer cells (spicules of Thomas) is longer and the processes are shorter because the wall is thinner. The substance of these casts was found to be rather less resistant to oxidation and alkali maceration than is ordinary cuticle, but far more resistant than the wall material; after moderate maceration it stains more deeply than does cuticle.

The preservation of these casts is most inconstant and unpredictable; well-preserved seeds from fruits may show them or lack them, and the same is true of isolated seeds. Then, too, the palisade cells alone may show them, or some of the fibrous cells alone, or they may be imperfectly preserved in one of these layers. The explanation advanced is that they are due to the post-mortem impregnation of the cells by oil which became fossilized as a resistant resin; other places which may show similar impregnation of cells are: the flesh of the fruit, the cells of the funicle, the interior of the megaspore, the mesophyll cells of the leaf *S. colpodes*, in all of which it is unpredictable whether a well-preserved specimen will show them. Of the seeds about a third show well-preserved casts, a third ill-preserved ones, and a third indistinct traces or none. Even better preserved casts occur in *C. thomasi*, but they are lacking in *C. nathorsti*.

The inner cuticle of the integument is exceedingly delicate; it is best preserved near the micropyle and becomes thinner still towards the base of the seed. It appears to be composed of small, elongated, straight-walled cells.

The space between the inner and outer cuticles of the integument in the compressed seed should give an accurate measure of the thickness of the integument. In *C. sewardi* it is 0.13–0.2 mm., in *C. nathorsti* 0.07–0.15 mm.

The micropylar canal is a tube about  $50\ \mu$  wide near the base, often widening above; the upper and lower surfaces of the tube are in contact in the compressed seed, and, as almost always with fossil cuticles preserved in contact with one another, they have stuck together making it impossible to separate the two sides. The appearance of the micropyle is not yet known from seeds compressed in other planes than the lateral, so that there is no indication whether the canal was round in section.

The canal is formed of a single layer of epidermal cells with a rather thin cuticle. This cuticle extends over their surface and also along their anticlinal walls, making the cell outlines very obvious indeed. The shape of the cells varies from isodiametric to about twice as long as wide; often they are elongated in the inner part and shorter in the outer part. Favourable specimens show clearly that one layer of surface cells alone is cutinized, but in some specimens where the anticlinal cutinized walls are distorted this is somewhat obscured. The canal often widens to about twice its width at the seed apex, and here the micropylar cells are continued in the lateral plane by the two tracts of very similar cells on the integument; in other planes the change in cell type occurs close to the opening of the micropyle.

No rock matrix is ever found in the micropylar canal, the sides of which are always pressed together and firmly adherent as is usual in compressions. This suggests that the canal was obliterated after pollination and before the seed was shed.

The micropyle is continuous below with the delicate cuticle of the inside of the integument which is adherent in the compressed fossil to the nucellus.

Comparison made between the micropylar canals of numerous seeds of *C. nathorsti* and *C. seawardi* led to the conclusion that there is very close agreement between them in essential structure and even in the shape of the cells. In *C. nathorsti* the canal is usually shorter (no doubt because the integument is thinner), but no general difference was recognized between the cells of the canal. Differences of interpretation will be apparent between this account and that by Thomas.

The nucellus is a thickly cutinized oval body and in seeds roughly treated in maceration alone survives. It is often a little broader in *C. nathorsti* (owing to its thinner integument); but no difference of structure can be recognized between them. In a good many seeds which appeared normal, some even showing pollen grains in the micropyle, the nucellus is thinly cutinized and has partially collapsed; presumably these seeds are of arrested development and abortive.

The cell outlines are conspicuous; the cells are large and rectangular in the middle of the seed, becoming smaller and shorter and finally square at the apex and base. At both the micropyle and chalaza there is a gap  $70\ \mu$  wide in this cuticle, and in seeds which have been roughly treated in maceration and have lost their micropylar canals the two ends appear just alike.

The megaspore membrane is of very varied development; at best it is a



thick densely staining sac extending up to the nucellus cuticle at the sides of the compressed seed. It usually shows strong irregular wrinkles concentric with the two ends of the seed; its substance is granular and non-cellular. Often it is much thinner, and in seeds with a poorly developed nucellus it is not seen. Like the material of the casts of the integument cells it is destroyed by oxidative and alkali maceration sooner than is ordinary cuticle.

Opposite the micropyle, the megaspore may be raised to form the structure called a 'plinth' by Thomas or it may be flat, or it may be sunken. These conditions are all seen both in *C. seawardi* and *C. nathorsti* as well as in *C. thomasi*.

### *Pollen in the micropyle.*

Search was made for pollen in the micropyle of these two species. At first it was seldom seen, but by improvements of technique it became possible to demonstrate it in about half of the well-preserved seeds. The difficulty is that when the oxidized seed is placed in alkali the substance of the integument dissolves, leaving the outer cuticle of the integument attached to the nucellus merely by the delicate micropylar canal which is seldom equal to the strains caused by movements in the fluid or by manipulation, so that it breaks and is lost or is so distorted as to make the recognition of pollen difficult. The following technique gives good results.

The seed is macerated as usual in  $\text{HNO}_3 + \text{KClO}_3$ , washed, and transferred to a slide. Nearly all the water is drained away and the minimum amount of ammonia is added. Droplets of fresh ammonia are added as needed and the spent liquid is dispelled by surface tension as a result of blowing over the seed a stream of alcohol vapour obtained from a modified wash bottle. When the maceration is complete, all the ammonia is dispelled; the seed is nearly dried in the alcohol vapour and it is quickly mounted in hot glycerine jelly. In this state the cuticles stick to the glass and so are protected.

This method is not fully applicable to seeds in fruits, as manipulation is needed to open the fruit and separate the seeds from one another; it was thus rather seldom that preparations suitable for demonstrating pollen were obtained. Pollen was seen in seeds of *C. seawardi* obtained from intact fruits; it has not yet been seen in *C. nathorsti* from fruits but has been seen in isolated seeds of the two species. The total number of preparations of seeds showing pollen is 38, of which 15 belong to *C. seawardi*, 16 to *C. nathorsti*, and 7 are undetermined.

The pollen grains are situated in the middle or more commonly at the base of the micropyle. They are usually very well preserved, and it was noted that in seeds showing them there are usually more than one, often three or four. All the grains were carefully examined and it was found that, without exception, they are of the winged Caytonanthus type. Usually this is obvious, but even in crushed specimens what can be seen was always consistent with the grain having been of this type. There is a possible slight difference between the pollen grains in the two species, but this is left for the present.

The occurrence of pollen in the micropyle of these seeds brings them into line with *C. thomasi*. Thomas (1934, p. 193) had indeed noted the presence of pollen in the micropyle of an isolated seed, but was inclined to attribute it to chance inclusion from the matrix; this explanation would not, however, explain its occurrence in so many seeds, particularly in ones from intact fruits. Though Caytonanthus pollen is common in the matrix it is less abundant than many other types of pollen and pteridophyte spore. It is very doubtful whether pollen would ever have got in in this way, as the absence of matrix in the micropyles of isolated seeds suggests that they were already closed at the time of preservation. In spite of their almost closed fruits the Caytoniales must be ranked as Gymnospermous in pollination; and no advance towards angiospermy was made between the time of the basal Liassic *C. thomasi* and these species of the Lower Oolites. The pollination mechanism is discussed later.

#### DISCUSSION OF THE MORPHOLOGY OF CAYTONIA

The essential form of the fruit, in so far as it is known at present, is well shown in Thomas' diagrams of sections of *C. sewardi* (Text-figs. 6, 7); that of *C. nathorsti* on sectioning proves to be the same. I had supposed that *C. thomasi* was different (see Harris, 1933, Text-fig. 10), but re-examination of the specimens has now convinced me that there is no difference except that the margin of the lip is bent upwards.

Thomas insisted rightly that the mouth of the fruit is the only discontinuity in the fruit wall and that it is tightly shut, and he based his theory of the pollination of the fruit partly on this fact; he did not, however, know of the occurrence of pollen in the seeds in the fruits, which was first found for *C. thomasi* (Harris, 1933) and now for the other species. This occurrence proves that the fruit must have possessed an opening of at least the size of the pollen grain—say  $30\ \mu$  in minimum diameter at the time of pollination—and there is nowhere for this opening to be situated but the mouth of the fruit. It is thus certain that the mouth was open at the time of pollination.

Harris in 1933 stressed the point that the pollen which reached the interior of the fruit also reached the seed since no stray pollen was found in the interior of a macerated fruit. This is equally true for the other species, but owing to the large amount of insoluble matter usually obtained it would be easier to miss a few grains. It is, however, true that very few grains can occur loose in the fruit.

This demands a mechanism which would serve to direct pollen grains to the ovules. Harris (1933) suggested that the fleshy wall of the fruit of *C. thomasi* formed pockets round the individual seeds leaving a narrow channel running from the mouth of the fruit to the micropyle of each seed. A pollination drop was supposed to catch and draw in wind-distributed pollen. This hypothesis is still held, though sections and broken specimens as *C. thomasi*

indicate that the seed pockets cannot have been as separate as indicated in the restored section (Harris 1933, Fig. 10); the evidence for the existence of separate channels running to each seed is, however, confirmed.

There is a very remarkable correlation between the structure of the mouth and the contents of the fruits: in *C. sewardi* there are about eight transverse bars and about eight seeds; in *C. nathorsti* about fifteen of each; in *C. thomasi* about thirty of each (in each case the numbers refer to well-developed fruits). This suggests that each bar might correspond to one seed. There is as yet no anatomical proof of a connexion, but it would seem possible that the delicate cutinized extensions from the 'stigma rays' (Thomas' term for the thickened bars in the cuticle of the mouth which he noted extending towards the seeds) might be of this nature.

The absence of any open channels through the mouth in the actual fossil now needs discussion. This may be explained as being the result of two processes; its obliteration during the later stages of fruit development and the obliteration of any remaining cavities by compression. The pressure of overlying rock is enough to flatten any air cavity in a delicate plant tissue; and it is a fact which is easily confirmed for these Yorkshire fossils that when two separate cutinized membranes were in contact at the time of preservation they adhere tenaciously to one another so that it may be difficult and is often impossible to get them apart. (This adhesion is shown by pollen grains on the surface of leaves and fruits and occasionally by the cuticles of two separate leaves.) This phenomenon then would account fully for the obliteration of any opening not supported by matrix; the existence in certain fruits of *Caytonia* of channels which do extend up for a short distance is probably accountable by their getting filled by matrix.

The peculiar barred structure of the mouth may now be considered. The explanation offered, which fits in with the converging inclination of the epidermal cells, is that the thick bars represent obliterated canals (Pl. VII, Fig. 11). While it is possible that the obliteration is entirely the result of preservation, it is likely enough that it occurred before death, in which case the thick middle zone which is only developed in the mature fruits represents the region where obliteration by cell growth occurred. Analogous obliteration of a channel after pollination is frequent or indeed normal in Gymnosperm seeds.

Although no really early stages of fruit development are known, something can be inferred from the series of stages present. As has been seen in the small fruits, it is the sac which is small; the rest is relatively normal, thus suggesting that the sac was late in enlarging. The arrangement of the cells on the wall of the sac in rows which curve round to the lip suggests that this is the real apex of the whole structure, and the following picture of early development is put forward:

(1) A strap-shaped organ developed a hollow in its upper surface just behind the apex. This stage is wholly imaginary.

(2) Growth at the sides and base of the hollow occurred in such a way as to form a sac, while bending at its point of attachment brought it into line with the stalk and approximated the former apex (now the lip) to the stalk.

(3) Concurrently with this, erect ovules developed in separate longitudinal furrows at the base of the sac; curvature of the sac caused them to be turned on to their sides, but they still face the mouth.

(4) The mouth of the sac is closed, apart from the furrows which remain as canals; the wall of the fruit becomes so fleshy as to obliterate nearly all the space inside.

(5) The sac and the ovules within grow considerably. At some stage at present unknown pollination occurs and the pollen grains falling on the lip are drawn through the canals down to the ovules.

(6) The canals are obliterated by cell growth; the seeds complete their development and their micropyles too are obliterated. There is little to show when fertilization took place, except that the occurrence of some pollinated seeds with a collapsed nucellus suggest that it was before full maturity.

So far the morphological discussion has dealt entirely with matters of the kind which, if *Caytonia* were a recent plant, would be demonstrated as observable facts. If, as is realized, this is based on inadequate evidence and is at best speculative, how much more doubtful must be the comparative morphology of *Caytonia*?

I believe no advance is yet possible on the simple view put forward by Thomas in 1925; namely that *Caytonia* is a megasporophyll showing pinnate branching, the pinnae bearing ovules on their incurved adaxial surfaces. *Caytonia* is thus closely comparable with the leaf *Sagenopteris* and the microsporophyll *Caytonanthus* of the same plant.

This view Thomas elaborated in the same paper, and still more in later papers when he brought the *Caytoniales* into closer comparison with flowering plants (1931) and with the *Corystospermaceae* (1933); but only by doing deliberate violence to prevailing ideas of morphology. Thus in 1925 he termed the individual megasporophyll segments 'carpels', while he later suggested that two or more segments or fruits correspond to a *Caltha* follicle or carpel. In 1934 the fruit was likened to the 'Cupule' of the *Corystospermaceae*, an organ of unspecified morphological nature: the whole female organ being compared on the one hand with the megasporophyll of *Caytonia*, and on the other with an inflorescence with bracts, bracteoles, and simple flowers as in the catkins of the *Amentiferae*.

In his work telome morphology was applied to seed plants. To me telomes and phyllomes are so to speak the atoms of comparative morphology into which all organs are analysable. To have to resort to telomes in morphological comparison is nearly the same as admitting that the organs in question seem very different. It is likely enough that new discoveries will show that organs which seem different can be quite simply related; but for the present no simple comparison can yet be made except perhaps between *Caytonia* and a

Pteridosperm megasporophyll. No one has claimed a genetic relation between Caytonia and the flowering plants; the contribution which they make towards the elucidation of the origin of that group is that by providing knowledge of plants in which pollination of almost closed fruits occurred, we may be helped to imagine earlier stages of the Angiosperms. It appears to me very possible that the styler canals of early fruits might have functioned in the same way as the canals through the mouth of Caytonia.

#### THE UNION OF THE GENERA GRISTHORPIA AND CAYTONIA

When Thomas described and distinguished the two Yorkshire species, he pointed out the great difficulty of deciding, in a strange group of plants, what differences were of generic and what merely of specific value; had the third species *C. thomasi* then been known in which the structure is intermediate, it would have afforded weight in favour of placing all three in one genus. In the present paper this is further supported, as it is shown that some of the supposed differences are inconstant.

The following statement summarizes the position.

*C. nathorsti* and *C. seawardi* agree in the organization of the megasporophyll, fruit, arrangement, and structure of the seeds. They differ in the dimensions of the different parts, the number of seeds, the shape of their epidermal cells of fruit and seed and in the thickness of the seed-coat. These differences appear to me exactly the sort which commonly separate allied species of a genus. Differences which have been shown to be inconstant and therefore of doubtful importance are the occurrence of a 'plinth' in *C. nathorsti* and the occurrence of well-preserved fibrous cells in *C. seawardi*. Other supposed differences are no longer believed to be real; namely the idea that *C. nathorsti* had a very large number of scattered ovules, and the idea that there were very considerable differences between the two seeds in structure.

Of the two possible names Caytonia is chosen because it is more fully described in Thomas' work; moreover, the name Caytonia was given in Thomas' preliminary note of 1912, whereas Gristhorpia was not mentioned until 1922.

#### THE FOLIAGE OF CAYTONIA SEWARDI AND C. NATHORSTI

Thomas (1925) gave evidence of agreement in structure and association for regarding Sagenopteris as the leaf of the Caytoniales; his view was not universally accepted, partly because the evidence was thought inadequate and partly because the two genera of fruits appeared to demand something more different than the two types of leaf which were suggested, though not fully distinguished.

Since then the evidence has been greatly strengthened. Edwards (1929) noted the association of *Caytonia nathorsti* and Sagenopteris in Sardinia; Thomas restated his evidence in 1931, and in 1933 Harris noted the striking association of an older species of Caytonia and Sagenopteris in two localities

in Greenland. Here further structural evidence is provided by the agreement of peculiar glandular hairs present on fruit-stalk and petiole.

The position for the Yorkshire species is now simplified by the recognition that the two fruits are species of a single genus, and that the associated *Sagenopteris* leaves are sharply divisible into two distinct species *S. phillipsi* (Brong.) *sensu-stricto* and *S. colpodes* Harris (1940).

The two species of leaf and the two species of fruit are distinguished from one another by several characters, the most reliable and constant of which is the shape of the epidermal cells. It has been found that there is remarkable correspondence between the epidermis of the fruits and of the upper sides of the leaves. *C. seawardi* and *S. colpodes* show cells with large and characteristic jagged thickenings, while *C. nathorsti* and *S. phillipsi* show cells with straight, thick walls and a characteristically bordered and sculptured inner area. The petioles and fruit-stalks show the same agreement too, but less strikingly than the leaf and fruit. When it is remembered that equally good agreement is shown by the fruit and leaf of the Greenland species, it will be seen that the structural evidence for their attribution is as perfect as it could well be. The microsporophylls *Caytonanthus* which I am convinced belong to the same group still need revision.

The following table gives the species of the Caytoniales.

Age.	Megasporophyll.	Leaf.	Microsporophyll.
Lower Lias	<i>Caytonia thomasi</i>	<i>Sagenopteris nilssoniana</i>	<i>Caytonanthus kochi</i>
Lower Oolite	<i>Caytonia seawardi</i>	<i>Sagenopteris colpodes</i>	<i>Caytonanthus arberi</i> belongs to one of these
Lower Oolite	<i>Caytonia nathorsti</i>	<i>Sagenopteris phillipsi</i>	

In addition there are many species of *Sagenopteris* ranging from Upper Trias to Lower Cretaceous; pollen (*Pityosporites*) more or less resembling that of *Caytonanthus* is widespread, and there are a few isolated seeds (*Amphorispermum*, Harris 1932) resembling those of *Caytonia*.

#### SUMMARY

1. The fruit and seed of *Caytonia seawardi* and *Gristhorpia nathorsti* are redescribed.
2. The presence of pollen in the micropyles of both is reported.
3. The structure which admitted the pollen is described and its nature discussed.
4. *Gristhorpia nathorsti* is renamed *Caytonia nathorsti* (Thomas) *n. comb.*
5. *Caytonia nathorsti* is referred to the leaf *Sagenopteris phillipsi* (Brong.); *Caytonia seawardi* is referred to *Sagenopteris colpodes* Harris.

I wish to acknowledge my debt to Mr. L. C. Willis for help with the photography, Mr. W. N. Edwards for valuable criticism of the manuscript, and to Mr. F. M. Wonnacott, the collector of the material, for freely given help in this work.

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#### EXPLANATION OF PLATE VII

Illustrating Professor T. M. Harris' paper 'Caytonia'

- Fig. 1. *C. sewardi* apex of seed showing pollen in micropylar canal (V. 26633).  $\times 60$ .
- Fig. 2. Same seed; note the folded nucellus and the two thick bands in the integument near the micropyle. The integument was damaged below in preparation.  $\times 30$ .
- Fig. 3. *C. nathorsti* somewhat immature seedless fruit showing the alternate thick and thin bars in the lower part of the mouth (V. 26655).  $\times 10$ .
- Fig. 4. *C. sewardi* surface of untreated seed (V. 26634).  $\times 150$ .
- Fig. 5. *C. nathorsti*, part of cuticle of the mouth showing zone 1 (here uniform) below, zone 2 dark, zone 3 with strongly marked thicker bars, above (V. 26656).  $\times 20$ .
- Fig. 6. *C. sewardi*, untreated seed (V. 26634).  $\times 20$ .
- Fig. 7. *C. nathorsti* untreated seed (V. 26657).  $\times 20$ .
- Fig. 8. *C. nathorsti* surface of seed (V. 26657).  $\times 150$ .
- Fig. 9. *C. sewardi* cuticle of mouth. In this photo zones 1 and 2 both appear black but the ridges in zone 3 are obvious (V. 26635).  $\times 50$ .
- Fig. 10. *C. sewardi*, inside of fruit wall from a specimen in which the cells of the flesh are preserved (V. 26636).  $\times 20$ .
- Fig. 11. *C. sewardi*, details of part of the specimen shown in Fig. 9  $\times 200$ .





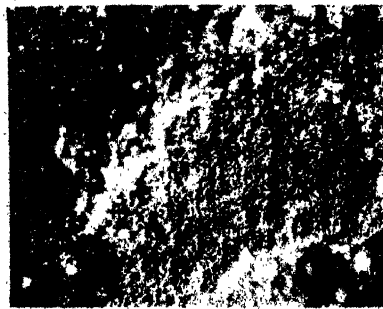




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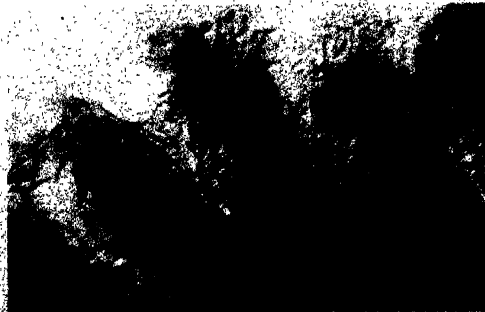


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# Studies in the Inheritance of Physiological Characters

## V. Hybrid Vigour in the Tomato

### PT. III. A CRITICAL EXAMINATION OF THE RELATION OF EMBRYO DEVELOPMENT TO THE MANIFESTATION OF HYBRID VIGOUR

BY

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With six Figures in the Text

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## INTRODUCTION

THE earlier papers in this series (Ashby, 1930, 1932, 1937; Luckwill, 1937) have contributed towards a further study of the problem of hybrid vigour as originally begun by Ashby in 1930. The notion of heterosis had hitherto remained somewhat vague, and might be summed up in the statement that the hybrid displayed a greater vigour of development. In an

attempt to give definition to this notion Ashby applied to this problem the methods of growth analysis initiated by Blackman (1919). He distinguished as factors contributing to the final size of the plant the embryo weight as the initial capital, and the relative growth rate as a measure of the rate of accumulation during the growth process. The greater size of the hybrid might be attributed either to a greater embryo weight or to a higher relative growth rate, and in his early experiments with maize hybrids (1930, 1932) Ashby produced evidence to show that in a cross between two varieties of maize, of which the parents differed in relative growth rate and in embryo size, the more vigorous vegetative development of the hybrid resulted from an embryo larger than that of either parent, while the relative growth rate of the hybrid was identical with that of the faster growing parent.

Later Ashby extended his studies to three crosses of tomato (Ashby, 1937). These experiments were concerned only with the early development of the plants up to the time of flowering of the first inflorescence, but Luckwill (1937), using another hybrid, examined more closely the stage of development subsequent to this, throughout flowering and fruiting. In this work with tomato the parental types as well as one of the hybrids were used but in no case were the reciprocal hybrids studied. In a further contribution Luckwill (1939) surveyed a wider range of tomato species and varieties including seven parental forms and twelve hybrids. In this case two reciprocal crosses were included. From his work Ashby again concluded that hybrid vigour was due to the maintenance of an initial advantage in embryo size and not to a relative growth rate higher than either parent, and in his first paper Luckwill (1937) agreed with him on this point. In his subsequent work, however, Luckwill (1939) modified this conclusion. He now found that embryo size was not invariably greater in the hybrid and suggested that 'the particular phase of the life-cycle of the plant at which weight heterosis is first exhibited varies considerably in different hybrids. It may first appear in the hybrid embryo, in the seedling, or in the young shoot primordium, or it may arise during the subsequent growth of the plant, either early or late in development according to the time of action of the factors involved.' It would appear, then, that the relation of embryo size to hybrid vigour remains obscure, and further work in the elucidation of this problem was undertaken.

In the course of the work here described it very soon became evident that embryo size is by no means a fixed character, since it is modified in response to the mode of pollination whether natural or artificial, to the environmental factors, to the position of the flower in the truss and on the plant, and above all to the genetic constitution of the maternal parent. In view of the complexity of the factors concerned in determining embryo size the problem of obtaining a representative and comparable sample of embryos of parents and of hybrids presents difficulties, and the conclusions drawn may be conflicting. Thus Whaley (1939*a, b*), using the same cross as Luckwill (1937), stated that the embryo size was intermediate between that of the parents, and not greater

than either, as the latter had consistently found. The first part of this paper deals with an analysis of the factors determining embryo size.

Apart from these considerations it was evident that a larger embryo size in hybrids presupposes either (1) a greater rate of development of the embryo between fertilization and the onset of dormancy, or (2) a variation in the stage at which the arrest of growth of the embryo at dormancy occurs. Both these possibilities were considered by Ashby, and at his suggestion an experimental investigation of this point was undertaken. In this paper the investigation of heterosis thus covers the stages of growth from fertilization up to full maturity of the plant.

## EXPERIMENTAL MATERIAL

The main experiments were confined to the study of two pure line types, both varieties of the ordinary tomato (*Lycopersicum esculentum*), and the reciprocal hybrids obtained by crossing the parental strains in both directions. The varieties used were: Line 107—a tall commercial variety 'Blaby', homozygous for the following factors D P O R Y S a h (Macarthur, 1934) and characterized by its large red fruit, simple inflorescences, and smooth light green leaves; Line 105—a dwarf variety, homozygous for factors d p o r y s A h and characterized by its small yellow pear-shaped fruit, compound inflorescences, and crinkled dark green leaves.

Throughout this paper the following notation is used:

Line 107, selfed . . . . .	77
Line 105, „ . . . . .	55
Hybrid 107×105, maternal parent 107 . . . . .	75
Hybrid 105×107, „ „ 105 . . . . .	57

## I. AN ANALYSIS OF THE FACTORS DETERMINING EMBRYO SIZE

### *Method of analysis.*

The very high positive correlation between embryo size and seed size in the tomato provides a method for following variations in embryo size without dissecting out the embryo. Examination of the mature seed shows the embryo tightly curled within a tough pellicle which in turn fits closely inside the hard woody testa. Since seed size provides such a good estimate of relative embryo size, the following analysis of seed weight was on a more extensive scale than any analysis of embryo weight could have conveniently been. Yet the results give a clear picture of the degree of variation in *embryo* weight as well, and allow the factors influencing embryo development to be analysed.

The parental fruit types, the large red fruits of 77 and the small yellow pear-shaped fruits of 55, can be regarded as associated with distinct seed sizes, the seeds of the former being much heavier than those of the latter. Seed size, then, is a varietal characteristic, and although the increase following cross pollination has been attributed to heterosis the possibility remains that the

causes of variation here are the same as those leading to variation of seed size in large and small fruits of the same plant, and not to the direct effect of the hybrid genotype.

Houghtaling (1935), examining the development of tomato fruits, found that their final size and shape are determined before pollination, and that enlargement after pollination is due to cell extension and is determined, therefore, to a high degree by the size of the individual cell. All the comparisons which Houghtaling made were between varieties. These variations in fruit size may be associated also with the sizes of seeds and their embryos. Houghtaling did not consider intra-varietal variation in fruit or seed size, both of which occur.

The normal development of tomato fruits depends on the success of pollination, and thus on the activity of growing embryos; if pollination fails the flowers drop, although occasionally very small 'snap' fruits develop in which no seeds are present. The final size of the fruit may thus be related to the number of ovules fertilized, and this again may affect seed size. The results of Gustafson (1939) would lend support to this view, for he found that normal fruits contain higher auxin content than parthenocarpic, and further he suggests that the auxin is largely derived from the pollen tubes during fertilization, the number of ovules fertilized thus determining the degree of extension growth of the fruit as well as the possible number of seeds.

The relations of fruit size, seed number, and seed size within a pure line were therefore experimentally investigated. Further factors considered were positions of fruit in the truss, position of truss on the plant, and environmental influences. The aim throughout has been to assess the importance of the genetic constitution of the embryo in determining its size as compared with the effects of the factors just enumerated.

#### *Variations in seed weight within the parent genotype 77 ('Blaby').*

The results of a study of the variation in seed size in this variety of tomato have recently been published by Luckwill (1939*a*) with which the present work is substantially in agreement. The results will therefore be presented only briefly.

In 1937 fruits were collected from six 77 plants growing under normal cultural conditions in a greenhouse. The fruits were picked as they ripened,

TABLE I  
*Statistical Constants of Distribution of Seed Number, Fruit Weight, and Seed Weight in Parent Type 77 ('Blaby')*

	Range of values.	Mean.	Standard deviation.	Coefficient of variation.
Seed number . . .	4-425	166±5	56	34%
Fruit weight (gm.) . .	7.32-143.25	48.7±1.45	16.44	34%
Mean seed weight . .	1.569-4.350	2.666±0.033	0.374	14%

the plant, the truss, and the position in the truss being recorded. Each fruit was weighed, and its seeds removed, dried, counted, and packeted. Altogether 128 fruits, collected over a period of two months from mid-August, were used for the analysis.

The frequency curves for 'fruit weight', 'seed number', and 'mean seed weight' appeared from inspection to be normal, indicating that variations were random and the sample of fruits a fair one. Statistical constants of the distributions are given in Table I. Seed number in individual fruits varied from 4 to 425 and there was a similar range of fruit weight, both distributions having a 34 per cent. coefficient of variation. Seed weight was not so variable (coefficient 14 per cent.), but the differences noted were comparable with any hitherto attributed to heterosis, and the widest range was equivalent to 100 per cent. of the mean.

TABLE II  
*Correlation Coefficients of Fruit Weight, Seed Number, and  
Seed Weight in Parent Type 77 ('Blaby')*

Factors correlated.		Total correlation coefficients.	Significance.
Seed No. (N) and Fruit Wt. (F)	$r_{NF}$	$+0.7501 \pm 0.0387$	S.
Seed No. (N) and Mean Seed Wt. (S)	$r_{NS}$	$-0.3272 \pm 0.0789$	S.
Fruit Wt. (F) and Mean Seed Wt. (S)	$r_{FS}$	$+0.1224 \pm 0.0871$	Not S.
Factors correlated.		Partial correlation coefficients.	Significance
Seed No. (N) and Fruit Wt. (F) eliminating Seed Wt.	$r_{NF \cdot S}$	$+0.8425 \pm 0.0257$	S.
Seed No. (N) and Mean Seed Wt. (S) eliminating Fruit Wt.	$r_{NS \cdot F}$	$-0.6383 \pm 0.0524$	S.
Fruit Wt. (F) and Mean Seed Wt. (S) eliminating seed Nos.	$r_{FS \cdot N}$	$+0.5886 \pm 0.0578$	S.

As seen in Table II, seed number was found to have a high positive correlation with fruit weight and to be negatively correlated with seed weight. There was no significant direct correlation between fruit weight and seed weight. The more seeds there are in a fruit, in other words the greater the success of pollination, the larger is that fruit, and the smaller are the seeds. The most probable interpretation of these results is that (1) the degree of fruit development is determined by the number of embryos growing and agrees with Gustafson's auxin theory of fruit development; (2) there is competition between individual seeds which increases as the number of seeds increases; (3) because correlation is not complete there must be other factors operating which can be related to the position of the fruit on the plant. For if the young fruit is stimulated to develop by the presence of embryos, the response by the fruit will depend upon its position on the plant.

In Table II the partial correlation coefficients of the factors are also presented. It is seen that on eliminating the effect of seed number there is a high



positive correlation between fruit weight and seed weight ( $r = 0.589 \pm 0.058$ ) which implies that with constant seed number a direct relationship exists between size of fruit and of seed, both being manifestations of a set of common causes.

For an individual fruit the interference of seed number can be eliminated by calculating the ratio 'Fruit Weight/Seed Number' (which will be called  $E$ ); when this is done  $E$  is found to be directly correlated with seed weight ( $r = 0.560 \pm 0.061$ ). The importance of the factor  $E$  is that among a group of fruits it indicates approximately the relative 'favourability' of conditions operating during development of embryos and seeds.

The size of tomato seeds within a pure line type, and hence the size attained by the embryos, is thus largely determined by the number of seeds in the fruit, and therefore by the effectiveness of pollination. The position of the fruit on the plant is of secondary importance, and its effects must be distinguished from variations in seed number with fruit position by the application of factor  $E$ .

#### *Position of fruit on plant.*

Luckwill (1939a) cites data on the effect on seed weight of number of fruits in the truss and the position of truss on the plant. The second factor, he concludes, has no effect, but seed weight decreases with increasing fruit number per truss as might be expected. From this it might be concluded that though there is competition between fruits in the same truss there is no competition between fruits of different trusses. This is contrary to the results of Gustafson and Laing (1931) who, by artificially reducing the number of fruits per plant, demonstrated competition within, as well as between, trusses. They found that pruning plants to one stem gave larger fruits though total yield was less, and that removing alternate inflorescences led to more numerous and larger fruits developing in the remaining trusses.

In the present study the number of fruits in the truss was not controlled, but it was observed that the position of the individual fruits in the truss did influence their development. The effect was a tendency for seed weight and  $E$  to fall in passing along the truss from the first to the last fruits, though the great irregularity in seed number masks this effect. Considering the first and last fruits of the 15 trusses from which all the fruits were collected, seed weight fell from 2.73 mg. to 2.42 mg. and  $E$  from 0.38 to 0.26.

Competition between trusses was demonstrated in the three lowest trusses of two of the plants. The first truss of one plant, A (Fig. 1), was forked and produced fourteen fruits compared with only seven on the simple first truss of the other plant, B. In consequence of the heavy demands of the first-truss fruits in the former, the factor  $E$  was greatly diminished for the second and third trusses, but in the latter, where the total demands of the first truss carrying seven fruits were much less, this was not evident. Seed size was correspondingly diminished in the case of plant A.

Another method of tracing the effect of position of the fruits on the plants was adopted. The data were rearranged in order of ripening of the fruits so that passing from fruits of the first truss to those of the last, and at the same time from first to last fruits within the individual trusses, it was observed that (1) seed number per fruit rises to a maximum and then falls, suggesting an optimum period for pollination during the flowering period; and (2) fruit

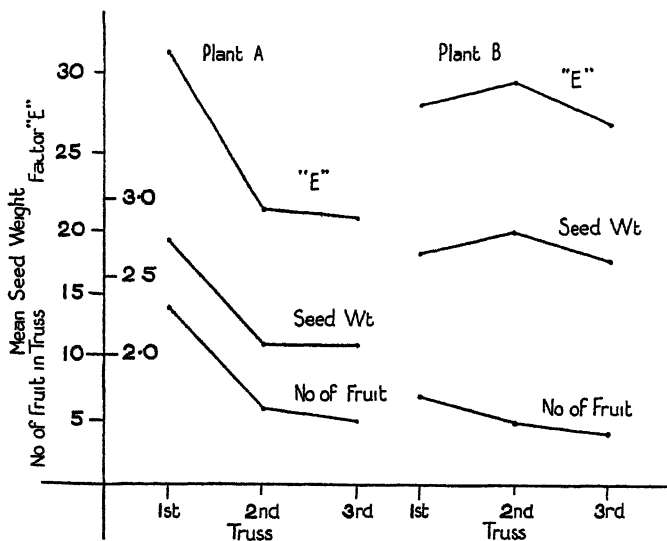


FIG. 1. Inter-truss effects on factor *E* and seed weight.

weight, *E*, and seed weight all tend to fall progressively, demonstrating the failure of supply to keep up with the increasing demands of the fruits. Further it was observed that the number of fruits per truss decreased from low to high trusses.

The position of the fruit on the plant therefore influences seed size but to a much lesser extent than variations in seed number. Contrary to Luckwill's conclusion there is competition between fruits both within and between trusses. Probably his finding was the indirect result of the method Luckwill adopted of controlling the number of fruits in the truss. He aimed at securing equal numbers of fruit in each group which necessitated using a larger number of trusses with few than with many fruits. Thus in his Table III (1939*a*, p. 183) he states that each entry represents the mean of six fruits, so that in the various classes of fruit number the number of trusses sampled must in the limit have varied inversely with the number of fruits per truss. Actually the number of trusses sampled in each class is nowhere stated. It may be assumed with certainty that more trusses were sampled in the class of low fruit number, and in such trusses the total demands on the plant's resources were much below normal, and this would reduce inter-truss competition considerably. It is probable too that *all* the trusses with high fruit

number were low ones, since the majority of these fruits were collected during the month of August, having ripened early. Thus in designing his experiment to investigate the effect of number of fruits in the truss, Luckwill invalidated it as a means of discerning an inter-truss effect.

TABLE III

*The Effect of External Environment on Seed Characters in  
Parent Type 77*

		Seed number.	Fruit wt.	Fruit wt. Seed no.	Seed wt.
Outdoor	. .	65	44 gm.	0.68	3.70 mg.
Indoor	. .	166	49 "	0.30	2.67 "

*The external environment.*

A random sample of ten fruits was taken from outdoor plants (77) and their weights, seed numbers, seed weights, and *E* values found. The factor *E* was clearly applicable, and a comparison of the outdoor and indoor data (Table III) solves the problem of seed size. The larger seeds of outdoor fruits resulted from the much lower number of seeds produced, *E* reflecting a similar difference. This simply means that in the open pollination is much less efficient.

TABLE IV

*The Relation of the Embryo Genotype to Fruit and Seed  
Characteristics*

Maternal parent.		107.		105.	
	Embryo genotype.	Selfed 77.	Hybrid 75.	Selfed 55.	Hybrid 57.
Seed No.	. . . .	207	143	87	49
Fruit wt. (gm.)	. . . .	101.4	92.6	26.4	18.9
Fruit wt.	. . . .	0.554	0.702	0.341	0.425
Seed no.					
Mean seed wt. (mg.)	. .	3.06	3.87	1.70	2.35

*The genetic constitution of the embryos.*

The following fruits were collected in 1938 from plants growing in a greenhouse. All fruits were derived from the second inflorescence.

From plants of pure line 107: (A) 10 hybrid fruit (genotype of embryos '75') resulting from artificial cross pollination; (B) 10 selfed fruit (genotype of embryos '77') resulting from natural self pollination.

From plants of pure line 105: (A) 10 hybrid fruit (genotype of embryos '57') resulting from artificial cross pollination; (B) 10 selfed fruit (genotype of embryos '55') resulting from natural self pollination.

The results of the analysis made are given in Table IV and in both cases

hybrid seeds were heavier than selfed seeds of the maternal parent. Other factors besides embryo genotype were, however, involved, for the *E* values were proportionately greater in the hybrids. Since the fruits of hybrid and parent developed on the same part of the plant at the same time, possible differences due to position on plant were reduced to a minimum; seed number is thus left as the probable determining factor. While hybrid seeds were obtained by artificial pollination, selfed seeds were obtained by natural pollination; and though each mode of pollination is largely uncontrollable, the former was found to be consistently less effective and gave fruits with fewer seeds, doubtless due to the limited application of pollen grains to the stigma. This reduction in seed number will account for all the size differences between hybrid and selfed seeds; in fact, in other fruits resulting from artificial pollination, no significant differences were found (Hatcher, 1939). With artificial cross pollination, quite apart from embryo genotype, there are thus two tendencies at work yielding larger seeds: (1) reduction of fruit number in the truss, and (2) reduction of seed number in the fruit, the latter being by far the most important. In the particular types studied one can, by selecting fruits, obtain at will hybrid seeds either significantly larger, or significantly smaller than the maternal parent seeds. Seed size, hence embryo size, appears to be such a variable characteristic as to mask any effects of hybrid vigour in the embryo unless care is taken to eliminate other factors in seed and fruit development.

## II. MANIFESTATIONS OF HYBRID VIGOUR IN THE RECIPROCAL

### HYBRIDS 75 AND 57

#### (i) *Embryo Development*

##### *Experimental procedure.*

The procedure was to kill and fix embryos at known ages of both parents (77 and 55) and of their reciprocal hybrids (75 and 57); these were dissected out, drawn, and measured. Growth curves were constructed and comparisons made between the different genotypes.

In the summer of 1937 about ten plants of each parent were available for the experiment; from these fruit of all ages, from 3 to 60 days after pollination as well as ripe fruits, were collected for each genetic type. Duplicate samples were always taken to enable true estimates of error to be made.

Normally in the tomato flower after self pollination the petals fold back when fertilization has occurred, 3 or 4 days after the bud opens. In this experiment all pollination was done artificially. The technique employed was to emasculate the flower just before the bud broke, removing with clean forceps the corolla tube with stamens attached. At the same time all open flowers were removed from the inflorescence, and this was protected in a special envelope against accidental pollination. Two days later the stigma,

then 'receptive', was dusted either with pollen from a flower of the same parent (selfed), or from a flower of the other parent (crossed). Such pollination was not always successful, for although ripe pollen can be recognized as being powdery the emasculated flower offers no guide as to the condition of the stigma. Nearly 200 pollinations were made in order to obtain the required fruits.

Fruits when collected were killed and fixed in Navashin's solution (24 hours), thoroughly washed in water, and preserved in 5 per cent. formalin.

A preliminary investigation was first made to obtain practice in dissection, and to ascertain the approximate embryo size at different ages. In the ripe seed the embryo was removed by employing dental chisels to cut the tough endosperm sheath so as to allow the curled embryo to slide out. The youngest embryo which could be isolated was 20 days old, and this isolation was accomplished by squeezing the seed under a coverslip and then searching for the embryo under the microscope; between these extremes were embryos of all sizes, demanding varying types of manipulation.

Since the younger embryos were much too small to be weighed the area was measured, which could be done at all stages of development. Though volume change would have been a better measure of growth, area provides a reliable estimate since the seed is flattened. The youngest embryo measured was a heart-shaped disc, and as the radicle and cotyledons developed these curled round into a flat spiral. With a camera lucida the magnified images of seeds and embryos were drawn, several combinations of objective and eyepiece being used with linear magnifications from 40 to 1,150 diameters. The areas of these drawings were determined with a planimeter and the absolute areas of seeds and their embryos calculated. Ten seeds from each fruit were measured with their embryos, this number being small enough to allow a margin for unsuccessful dissections, yet large enough to give a reasonable estimate of mean area per fruit.

### *Experimental results.*

Tables V and VI summarize the results of the area determinations of seeds and embryos. The following conclusions as to the variabilities of the samples were reached: (1) the embryos were more variable than the seeds, but this difference tended to disappear as dormancy approached; (2) fruits, hybrid and selfed, from parent 77 were less variable in seed and embryo size than fruits from parent 55; (3) hybrid seeds and embryos were less variable than seeds and embryos of their maternal parent. The results are presented graphically in Fig. 2.

The large variation in size of young embryos, for in a fruit of 20 days old one embryo may be twice the size of another, suggests that there is no precise time of fertilization and that earlier zygote division by itself is unlikely to be the cause of large hybrid embryos, which Ashby suggested as a possibility.

TABLE V  
*Cross-sectional Area of Seeds and Embryos at Various Times after Pollination in Parent Strain 77 and Hybrid 75 (Maternal Parent 77)*

**D = days from pollination; A and B are replicate samples.**

D	Parent 77.				Embryos.				Hybrid 75.				Embryos.			
	Area sq. mm.	Standard error.	Coeff. of variation.	Area sq. mm. × .01.	Standard error.	Coeff. of variation.	Area sq. mm.	Coeff. of variation.	Area sq. mm. × .01.	Standard error.	Coeff. of variation.	Area sq. mm.	Coeff. of variation.	Area sq. mm. × .01.	Standard error.	Coeff. of variation.
20	A 2.49	0.053	6.74	5.25	0.307	18.51	2.74	0.086	9.93	5.41	0.344	20.13				
	B 2.59	0.082	9.97	5.76	0.421	23.13	2.67	0.087	10.32	4.59	0.189	13.05				
25	A 2.84	0.140	4.93	7.80	0.786	31.88	5.74	0.076	4.20	31.18	1.88	16.43				
	B 2.92	0.086	9.34	8.60	0.379	13.94	5.35	0.113	6.67	24.48	0.690	8.92				
30	A 6.33	0.195	9.76	40.96	1.96	15.09	8.91	0.149	5.30	128.70	6.17	15.17				
	B 6.39	0.215	10.67	43.96	1.07	7.72	8.28	0.164	6.28	121.23	2.94	7.66				
35	A 8.47	0.167	6.23	184.29	7.19	12.35	10.42	0.139	4.21	235.95	8.61	11.55				
	B 10.75	0.179	5.26	229.80	7.20	9.91	10.50	0.253	7.61	217.33	6.24	9.07				
40	A 9.38	0.180	6.07	147.50	6.49	13.92	9.88	0.160	5.12	275.54	4.60	5.28				
	B 8.75	0.187	6.76	166.76	6.05	11.47	11.27	0.179	5.02	313.60	6.29	6.34				
50	A 13.22	0.285	6.81	315.62	8.78	6.89	15.35	0.287	5.92	409.83	10.43	8.05				
	B 16.55	0.436	8.33	438.95	15.96	11.50	13.67	0.336	7.77	346.71	7.95	7.25				
60	A 14.60	0.294	6.36	393.12	11.58	9.32	15.31	0.371	7.66	393.16	12.08	9.71				
	B 14.54	0.222	4.84	394.94	10.81	8.65	15.75	0.262	5.25	373.98	10.19	8.61				
Mature	A 11.27	0.447	12.54	379.55	12.38	10.31	12.77	0.213	5.26	410.73	8.98	6.92				
	B 12.04	0.163	4.29	376.28	6.57	5.52	13.09	0.302	7.29	378.36	8.18	6.84				

TABLE VI  
*Cross-sectional Areas of Seeds and Embryos at Various Times after Pollination in Parent Strain 55 and Hybrid 57*  
 (Maternal Parent 55)

D = days from pollination; A and B are replicate samples.

D	Parent 55.				Hybrid 57.			
	Seeds.		Embryos.		Seeds.		Embryos.	
	Area sq. mm.	Standard error.	Coeff. of variation.	Area sq. mm. × 0.01.	Area sq. mm.	Coeff. of variation.	Area sq. mm. × 0.01.	Coeff. of variation.
20	A 1.64	0.049	9.42	2.33	1.83	0.054	4.96	0.263
	B 1.30	0.056	13.68	1.66	1.91	0.060	6.33	0.504
25	A 2.94	0.095	10.22	12.66	2.21	0.040	11.98	0.601
	B 3.17	0.111	11.08	11.00	1.75	0.067	7.26	0.558
30	A 2.73	0.102	11.84	11.36	3.80	0.087	48.47	4.35
	B 3.01	0.077	8.11	14.54	3.26	0.095	32.82	2.67
35	A 3.65	0.113	9.82	50.52	4.45	0.134	99.55	8.20
	B 4.21	0.155	11.67	72.40	4.45	0.107	154.32	5.01
40	A 3.60	0.121	10.36	71.27	6.39	0.160	203.67	7.75
	B 3.48	0.097	8.83	49.26	4.69	0.098	149.52	4.34
50	A 4.29	0.265	10.53	130.34	5.76	0.113	225.12	3.99
	B 7.52	0.434	18.25	210.69	6.17	0.228	230.92	8.25
60	A 7.94	0.236	9.39	211.89	7.64	0.211	197.87	3.16
	B 6.87	0.126	5.81	183.55	8.98	0.186	245.01	5.85
Maternal	A 7.12	0.192	8.51	272.92	7.26	0.260	269.39	9.19
	B 7.68	0.238	9.78	291.63	8.29	0.182	288.04	5.98

The smaller variations of seeds and embryos in fruits from 77 plants compared with 55 may be related to the shape of the fruits and the spatial relations of the ovules.

The fact that hybrid seeds and embryos were less variable than those of

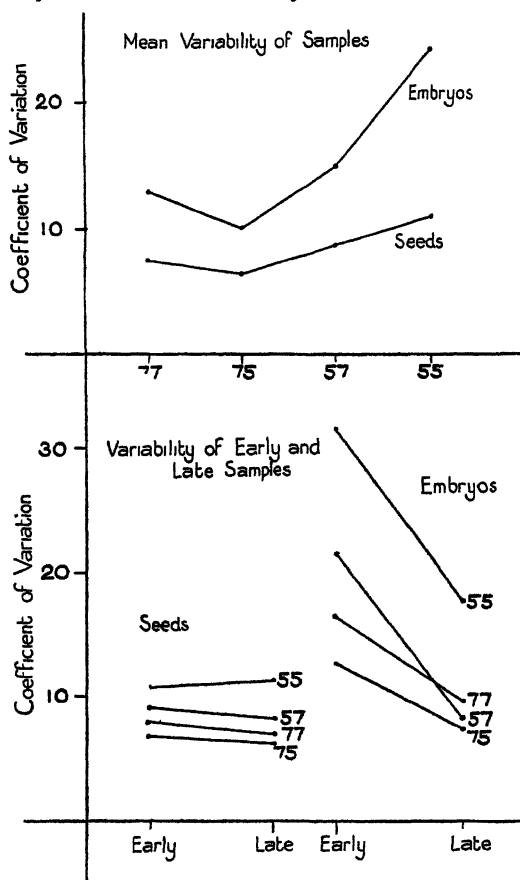


FIG. 2. Variability of seed and embryo samples.

their maternal parent suggests that hybrid embryos were more active in obtaining nutrient supplies. In the parent 55, but not in the hybrid 57, aborted seeds were found with the embryos still in the primary stage immediately preceding the rapid development of the cotyledons.

*Embryos.* In Fig. 3 are plotted the curves of relative growth in area for the four genotypes, the two parents 77 and 55 and their reciprocal hybrids 75 and 57. From days 20 to 35 the embryos grew rapidly, followed by a period of falling growth to day 50, after which further growth was comparatively small. At day 50 the fruits were still green so that embryo growth had been completed before the fruit ripened.



To test for significant differences between the individual growth curves the method of variance analysis (Fisher, 1938) was used. In the primary analysis the variances due to strain, time, and strain-time interaction were all found to be highly significant, and further analysis of these variances were

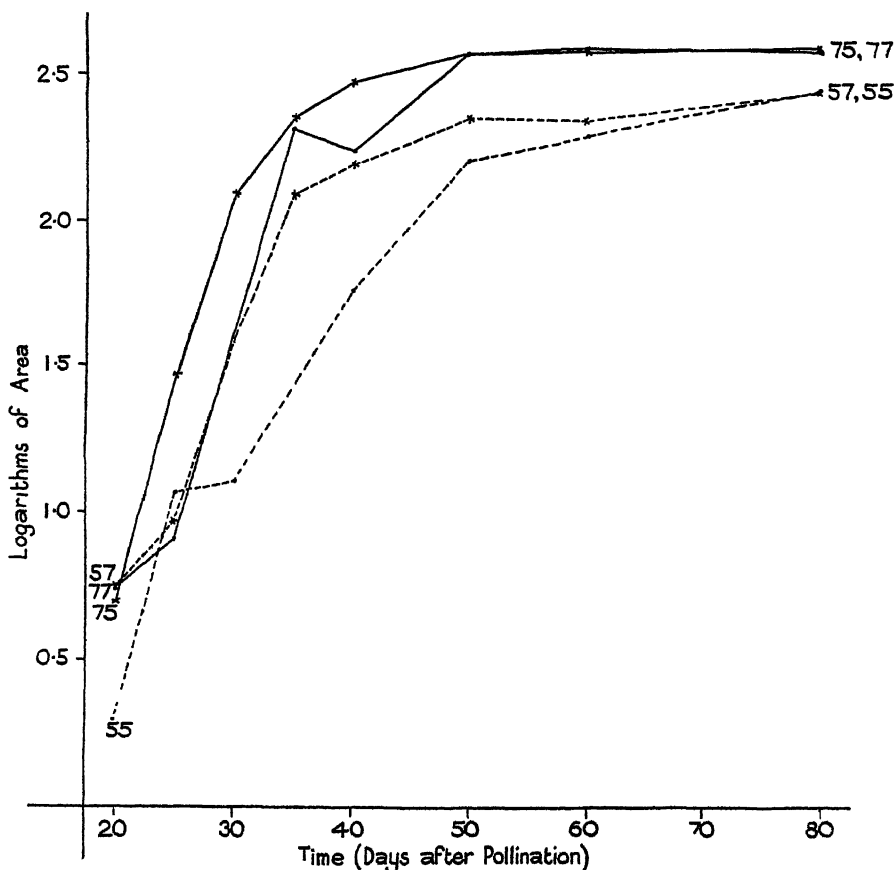


FIG. 3. Growth of embryos. Logarithms of areas plotted against time. Continuous lines maternal environment 77, broken lines maternal environment 55. Circles parent, crosses hybrid.

carried out into (1) the three phases of growth from days 20–35, 35–55, and day 50 to maturity respectively; (2) comparisons of pairs of strains; (3) comparisons of pairs of strains in each phase of growth. The results of this analysis are summarized in Table VII.

Time variance is highly significant ( $P = 0.01$ ) up to day 50, but in the last phase of growth is reduced to the 5 per cent. point. In the comparisons of strains this variance is insignificant in three cases out of four, showing that after day 50 growth was almost completed. For the first and second phases of growth, variance due to strains is significant for all comparisons, hybrid

embryos being larger than their maternal parent embryos, thus manifesting size heterosis. Parent 77 was larger than parent 55, and correspondingly hybrid 75 exceeded hybrid 57 in size. In the final growth phase, after day 50, there is no significant strain variance in either comparison involving hybrid

TABLE VII

*Summary of Statistical Analysis of Cross-sectional Areas of Embryos (Logarithms)*

o variance less than experimental error.  
— variance not significantly greater than error.  
+ 5 % significance by 'Z' test ( $P = 0.05$ ).  
++ 1 % significance by 'Z' test ( $P = 0.01$ ).

Variance due to	Develop-ment stage.	Strain comparison.				
		77 & 75.	75 & 57.	57 & 55.	77 & 55.	All strains.
Time	Day 50-maturity	o	—	+	—	++
	Day 35-50	++	+	+	++	++
	Day 20-35	++	++	++	++	++
	Day 20-maturity	++	++	++	++	++
Strain	Day 50-maturity	—	++	—	++	++
	Day 35-50	+	++	++	++	++
	Day 20-35	++	++	++	++	++
	Day 20-maturity	++	++	++	++	++
Time and strain inter-action	Day 50-maturity	o	—	—	—	—
	Day 35-50	+	o	—	o	—
	Day 20-35	++	+	+	++	++
	Day 20-maturity	++	++	++	++	++

and maternal parent, showing that the size of the mature embryo, whether selfed or hybrid, was determined by the environment in which it developed, and the heterosis present during embryo growth was no longer evident. The embryos associated with the maternal environment of 77 were significantly larger than those associated with parent 55.

Considering variance due to interaction of strain and time highly significant differences ( $P = 0.01$ ) were found in all strain comparisons, but when further divided into the three phases of growth this significance is confined to the rapid period of growth from days 20 to 35. As a result of these growth-rate differences size heterosis was manifested in both hybrids, but to different degrees. The results are not extensive enough to draw definite conclusions as to the exact time of initiation of hybrid vigour, but at 20 days types 75, 57, and 77 were similar in size, suggesting that at this stage heterosis was not present. Embryos of 57 were never larger than those of the parent 77, and the differences between the reciprocal hybrids, first apparent at about day 25, were maintained throughout subsequent development. At the end of the growing period, i.e. in the mature fruit, the hybrid embryos 75 and 57 thus represent different phenotypes of the same genotypes, and their size difference is not

due to heterosis but is a result of the environmental conditions within the ovaries of the genetically distinct maternal parents.

*Seeds.* A similar analysis for seeds was carried out and the results showed that their development runs closely parallel with that of the embryos. Growth was again negligible after 50 days, and in final size the hybrid seeds were the same as those of the maternal parent. Growth-rate differences were significant only in the period of rapid growth, the hybrid seeds then showing heterosis like the embryos.

*Developmental growth.* The main developmental changes take place during the phase of growth between 20 and 35 days. At day 20 the embryo is in the primary condition with cotyledonary rudiments and a heart-shaped appearance; sometimes the suspensor is still visible. There follows rapid growth of the cotyledons and radicle to which most of the size increase of the embryo is due, and the embryo now assumes its familiar coiled condition within the endosperm sheath. The plumule does not develop and is seen in the mature embryo as a dome-shaped mass.

At 20 days embryos of types 75, 57, and 77 were all at the same stage and of the same size. The embryos of 55 showed no rudiments and in consequence were smaller. This suggests that at this early stage size heterosis was not present, and also that differences in the hybrids due to maternal environment had not appeared. For some time after this the embryos displayed the differential growth rate of the four genotypes, but the interaction of genotype and maternal environment is evident in the differences in size established in the reciprocal hybrids at 25 days and thereafter maintained. Ultimately the genotypic effect on growth rate disappeared and the final embryo sizes were determined solely by the maternal environment, through limitation of embryo growth by the testa which, although stimulated to develop by the presence of an embryo, attained a pre-determined maximum size.

The conclusions which may be drawn from the preceding discussion are as follows: (1) The size of the mature embryo is determined wholly by maternal environment, for genotype 75 resembles 77, and 57 resembles 55. (2) At comparable times during their development the hybrid embryos are larger than embryos of their maternal parent, for 75 is greater than 77, and 57 is greater than 55. (3) During development the particular maternal environment influences the size of the embryos with the result that reciprocal hybrids differ significantly, for except in the primary stage 75 is much larger than 57. (4) Embryos of type 57 never exceed in size the embryos of the tall parent 77. It follows that to claim differences in size as the basis of heterosis effects in the embryo is illegitimate, since the environments in which the embryos develop are dissimilar. During embryo growth the only test of heterosis is for the embryo to be greater in size than that of its maternal parent, and both reciprocal hybrids should show this advantage. In post-germination growth the definition of heterosis as understood in this paper is that character of the hybrid plants by virtue of which they exceed both their parents in vigour of growth.

(ii) *The Vegetative and Flowering Periods*

*Experimental procedure.*

In 1938 the four embryo types were grown to maturity to determine the magnitude of hybrid vigour in the different periods of the life-cycle, and to observe the influences on subsequent growth of the different conditions prevailing during embryo development as provided by the maternal parents of the reciprocal crosses. The layout of the experiments consisted of two blocks within which each of the four genotypes was arranged in a purely random manner. Although of the hybrid and parent 77 numerous plants were available, owing to poor germination in the parent 55 the maximum number of plants which could be used was six. In each block, therefore, each genotype was grown in triplicate.

The seeds of the reciprocal crosses differed considerably in weight, and from the parent types two groups of seed were selected, one of equal size to that of the hybrid, the other smaller. Thus of parent 77 one set of seed was equal in size to that of the hybrid 75, the other smaller: the same for types 55 and 57. On March 23 the seeds were sown singly at a depth of  $\frac{1}{4}$  in. in 3-in. pots to ensure minimum disturbance at planting out.

On May 3, 41 days from sowing, the plants were planted out in the greenhouse plot, and were allowed to grow freely for nearly four months. During the two days Aug. 29–30 on all the plants the following observations were made: (1) height of plant; (2) total fresh weight above ground; (3) weight of main shoot with laterals and fruits removed; (4) weight of green and ripe fruit; (5) thickness of main stem at base; (6) number of internodes, leaves, and inflorescences on main shoot; (7) length of each internode; (8) fresh weight of lateral shoots with fruits removed; (9) total length of lateral shoots.

In 1939 the experiment was repeated on a larger scale, when 12 of each genotype were eventually grown to maturity. Only new data are here included, chiefly observations on seedling development, as otherwise the results confirmed entirely those obtained in 1938.

*Experimental results—1938.*

Germination rate was intermediate in the hybrids, on the average nearly a day later than the tall parent 77. Four weeks after germination the 77 plants were furthest developed, seven leaves had unfolded, the first flower-buds were visible, and these plants were the largest of all the types. The hybrids 75, 57, resembled parent 77, but were less advanced and slightly smaller. Only six leaves had unfolded, and flower-buds were not visible. Reciprocal hybrids could scarcely be distinguished, either from each other or from parent 77. The other parent, 55, was markedly dwarf. At this stage there was no hybrid vigour manifest, but when the plants were measured nearly 23 weeks after sowing the hybrids showed size heterosis to a remarkable degree, and both 75 and 57 were taller and heavier than either parent.

**Height.** Height is not always a good criterion of hybrid vigour but expressed as a function of development is a valuable indication of structural size. The height of a plant is determined by the number and length of the internodes composing its main stem, and either may be responsible for heterosis. In the hybrids 75 and 57 there were no more internodes than in parent 55 but the greater length of the internodes resulted in plants 40 cm. taller. Parent 77, which at 40 days exceeded all others in height, was at the end of the season the shortest—with 5 leaves less and with a mean internode length of 3.45 cm. compared with 3.85 cm. of parent 55, and 4.80 cm. of the hybrids. More important than final height is the variation in internode length along the stem, and the height data have been analysed further in order to compare the plants at equivalent *stages* of development. In Fig. 4 are plotted logarithms of height for all strains at the insertion of every 5th leaf up the stem. Each point plotted is the mean of 6 values (triplicate samples in each of two blocks) and the results for heights up to leaf 25 have been analysed statistically. (The analysis could not be extended to leaf 30 since parent 77 produced on an average only 29.5 leaves.) By this method height differences were localized.

TABLE VIII

*Summary of Statistical Analysis of Height (Logarithm)  
Measured at Comparable Developmental Stages*

○ variance less than experimental error.  
— variance not significantly greater than error.  
+ 5 % significance by 'Z' test ( $P = 0.05$ ).  
++ 1 % significance by 'Z' test ( $P = 0.01$ ).

Variance due to	Development stage.	Strain comparison.					
		77 & 75.	75 & 57.	57 & 55.	77 & 55.	77 & 57.	75 & 55.
Strain	Leaf 5-10	—	—	+	+	○	○
	„ 10-15	○	—	++	++	+	++
	„ 15-20	+	—	++	++	++	++
	„ 20-25	++	○	++	++	++	++
	„ 5-25	○	—	++	++	+	++
Development-strain interaction	Leaf 5-10	—	○	—	○	—	—
	„ 10-15	○	○	○	○	○	○
	„ 15-20	○	○	○	○	○	○
	„ 20-25	—	○	○	—	—	○
	„ 5-25	+	—	+	—	+	+

Statistical analysis showed that significant differences ( $P = 0.01$ ) occur in the variances due to development, strain, and their interaction. This means that different heights were attained in the different strains, and that growth in height at various developmental stages differed also among the strains. Strain and interaction variances were further subdivided and comparisons made between the genotypes in pairs at successive periods of development, as shown in Table VIII.

There are significant differences among strains in all comparisons except between the reciprocal hybrids 75 and 57, although in their early development from the 5th to the 10th leaf these hybrids do not differ from the parent 77. Interaction variances, either between the parents 77 and 55 or between

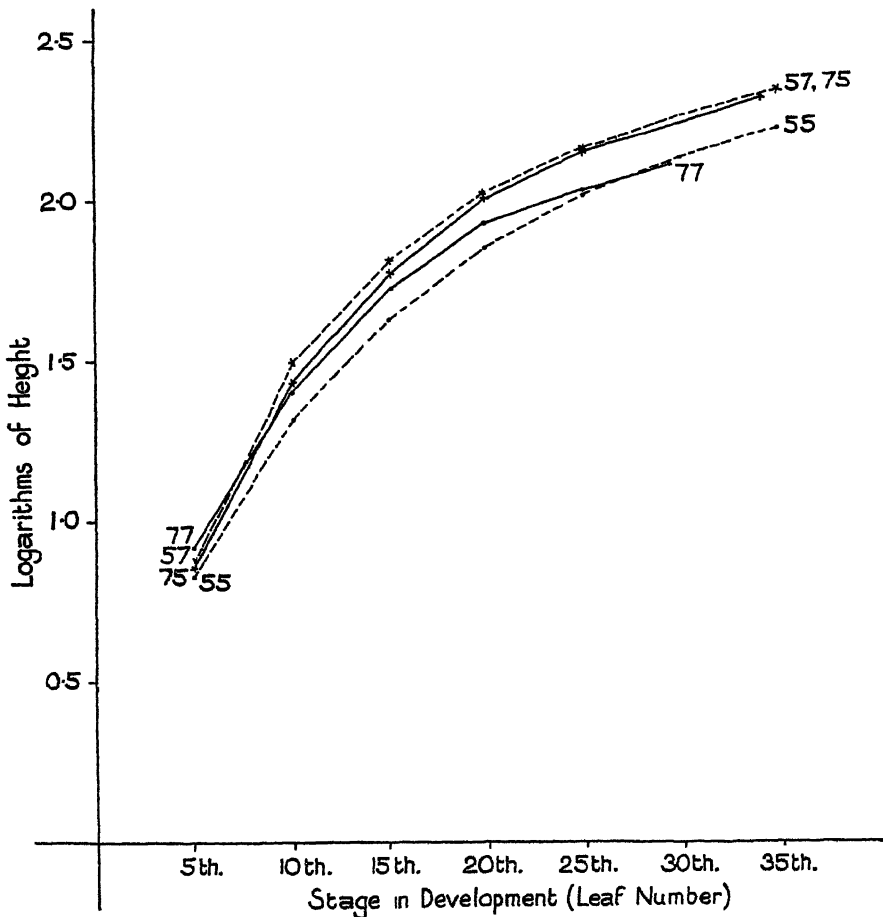


FIG. 4. Logarithms of height plotted against stage in development. Continuous lines: circles parent 77, crosses hybrid 75. Broken lines: circles parent 55, crosses hybrid 57.

hybrids 75 and 57, are not significant. In the comparisons between parent and hybrid the interaction variances are significantly indicative of development rates higher in 75 and 57 than in 77 and 55. When these variances are partitioned into the different development stages none of these is significant, yet they nevertheless serve to indicate the periods of major differential growth. Rate differences are apparent in all comparisons involving hybrid and parent, but are restricted to the region up to the 10th leaf, and to the region after leaf

20 in comparisons in which parent 77 is concerned. The meaning of this is apparent from an inspection of the height curves (Fig. 4).

At the 5th leaf stage the hybrids were intermediate in height between the parents. At the 10th leaf, however, the hybrids had gained in height on the

TABLE IX  
*Heterosis in Mature Hybrids*

Strain.	Parent 7 × 7.	Reciprocal Hybrids.	Parent 5 × 5.	
		75.	57.	
Embryo size.	Small.   Large.	Large.   Small.	Small.   Very small.	
1. Mean seed weight (mg.)	2.64   3.31	3.44   2.01	2.08   1.11	
2. Height (cm.)	131   127	207   216	161   165	
3. Mean internode length	3.5   3.4	4.7   4.9	3.9   3.8	
4. Leaf no. on main stem	29.5   29.5	34.2   35.0	33.5   36.5	
5. Leaf no. before truss A	7.6	8.7	9.3	
6. " " " B	3.5	3.6	4.8	
7. " " others	3.0	3.0	3.7	
8. Inflorescence no. (main)	7.6	9.0	7.2	
9. Thickness of stem base	1.01	1.37	1.22	
10. Total length of laterals	198   223	733   952	747   643	
11. Total fresh weight	1390   1525	3104   4025	1725   1792	
12. Weight main stem	219   225	396   461	325   400	
13. Weight laterals	146   169	700   1061	875   692	
14. Inflorescence type	Simple	Simple	Compound	
15. Fruit characters	P.O.R.Y.	P.O.R.Y.	p.o.r.y.	
16. Mean wt. of ripe fruit	47.5	38.0	15.8	
17. Total wt. of fruit	1085	2241	621	
18. Ratio ripe/green	1.53	0.68	0.10	
19. " fruit/laterals	6.71	2.68	0.93	

parents, and this advantage was maintained throughout further growth. From leaf 10 to leaf 20 all the curves are parallel. After leaf 20 the curve for parent 77 flattens off more abruptly than any of the other curves. The following points are noteworthy: (1) the reciprocal hybrids 75 and 57 attain the same height and do so at the same rate, and thus manifest equal degrees of height heterosis; (2) height heterosis was initiated by a greater development rate between leaves 5 and 10 and was then maintained; (3) for a long period the curves are parallel, showing that height increased at the same relative rates in all types; (4) relative rate of height increase progressively declined with development, most severely in parent 77.

*Weight heterosis and fruiting.* There was considerable weight heterosis in

the hybrids, and in Table IX, which summarizes all the observations made on the mature plants, the relation to the parental types is shown. Comparing first with parent 77 it is seen that weight heterosis was due to the production by the hybrids of more fruit, more main shoot growth, and more lateral growth. The dwarf parent 55, in strong contrast, produced about as much main shoot growth and lateral growth as the hybrids, and heterosis was due almost entirely to fruit production.

The earlier or later initiation of the flowering phase, the prelude to fruiting, depends in the tomato on the number of leaves produced before the first truss, and its progress on the number of leaves between each successive inflorescence. In this respect the hybrids 75 and 57 were actually intermediate, the leaf number before the first truss being 8·7, compared with 7·6 in parent 77 and 9·3 in parent 55. In addition to this leaf difference the parent 77 had a slight advantage in development, having germinated first; this resulted in the earlier ripening of fruits. In the spacing out of subsequent inflorescences it is to be noticed that while an interval of three leaves was the rule in parent 77 and in the hybrids, five leaves frequently occurred between inflorescences of parent 55, the result being to the advantage of the vegetative system. The incidence of the fruiting phase was earliest in parent 77, intermediate in 75 and 57, and progressively later in parent 55 which produced on the main stem two trusses less than the hybrid though the number of leaves was the same. Parent 77 produced fewer trusses of fruits, and also fewer leaves.

The size of individual fruits was also intermediate in the hybrid plants, though these, being heterozygous for the dominant fruit character of parent 77, produced the round red fruit of this parent. The much larger number of fruits in the compound inflorescences of parent 55 weighed considerably less than the smaller number of fruits on the simple inflorescences of the other types, and thus the diversion of nutrient materials interfered less with vegetative development. The ratios of total fruit weight to total lateral weight thus fell from 6·71 in 77, to 2·68 in 75 and 57, and 0·93 in 55. Such a result for tomato confirms the active competition by the fruits for nutrient supply as found by Gustafson and Stoldt (1936) and Gustafson and Houghtaling (1939). Since the fruits exert the heaviest demands (for they contribute most to the total weight) the larger the fruit the greater the proportion of nutrient material needed to supply them, and the smaller the lateral development. Again more fruits developed in the inflorescences of the hybrids compared with parent 77 because the supply gradient between the smaller fruits was less steep. Thus parent 77 approached senescence rapidly and its vegetative growth was restricted (lateral and main shoot) while the fruits ripened early. The hybrids and parent 55 continued their vegetative growth longer, with the result that heterosis only appeared in relation to the fruits, whereas compared with parent 77 the heterosis was found in the hybrids in relation both to fruits and vegetative growth.

The establishment of height heterosis in the hybrids between the 5th and



10th leaf stage is also a direct result of the fruiting relations, for not till this stage in growth does the differential behaviour of the strains with respect to laterals and fruit production begin to operate. In the hybrids 75 and 57 the manifestations of hybrid vigour after germination was related chiefly to the fruiting characters of the plants and, as is shown clearly in Table IX, results from the combination in the hybrids of the favourable characters of both parents. Considerable heterosis was evident even though the parent 77 is almost 'top dominant', due to the interplay of the correlative physiological factors.

*The effect of embryo size.* The different genotypes have so far been considered without reference to initial embryo size. Plants were, however, grown from small and large embryos, selected within a pure line, while the reciprocal hybrids provided a natural variation in size. In the height analysis the reciprocal hybrids did not differ significantly, and the curves show the 57 hybrid plants from small embryos to have been taller if anything, even at the 5th leaf stage, than the 75 hybrid plants from large embryos. When the parent types were grouped according to embryo size, no systematic effect was apparent. In the observations on the mature plants (Table IX) the differences between plants from small and large embryos, either within a parent type or between reciprocal hybrids, were unimportant compared with the striking differences between the genotypes. Embryo size was, in fact, not important in determining final size of the plants, but may have influenced the early seedling stages.

#### *Experimental results—1939.*

The results of the 1939 experiment confirmed entirely those of the previous year; in addition some observations were made on seedling growth and on leaf development. Measurements were taken of hypocotyl and cotyledon lengths from the first day of germination to the completion of their growth, and these were used to construct the relative growth curves of Fig. 5. The development of both hypocotyl and cotyledons was influenced by the germination rates, but it is evident that their final size was determined partly by genetic constitution and partly by the maternal environment of the embryo. The dwarfness of 55 was very obvious, while in hypocotyl and cotyledon size the hybrid 75 (maternal parent 77) exceeded its reciprocal hybrid 57 (maternal parent 55). In cotyledon lengths the relations are the same as in the period of rapid development of the embryo, with 75 the greatest, 77 and 57 approximately equal, and 55 much smaller. The hybrid 75 did not exceed parent 77 in hypocotyl length, but since the hypocotyl is an ill-defined region no comparison can be made with embryo development. Size heterosis, present in the developing embryo but masked in the mature seed, was thus re-established; the major effect on the cotyledons conforms with the fact that they constitute the main bulk of the embryo. Embryo size affect, therefore, the growth of the hypocotyl and the cotyledons in the post-germination period.

Measurements made on the development of the 1st, 5th, and 10th leaves have been used to construct relative growth rate curves (Fig. 6). The first leaves of parent 77 and the reciprocal hybrids 75, 57, were of the same size, showing that there was no effect of maternal environment; no size heterosis

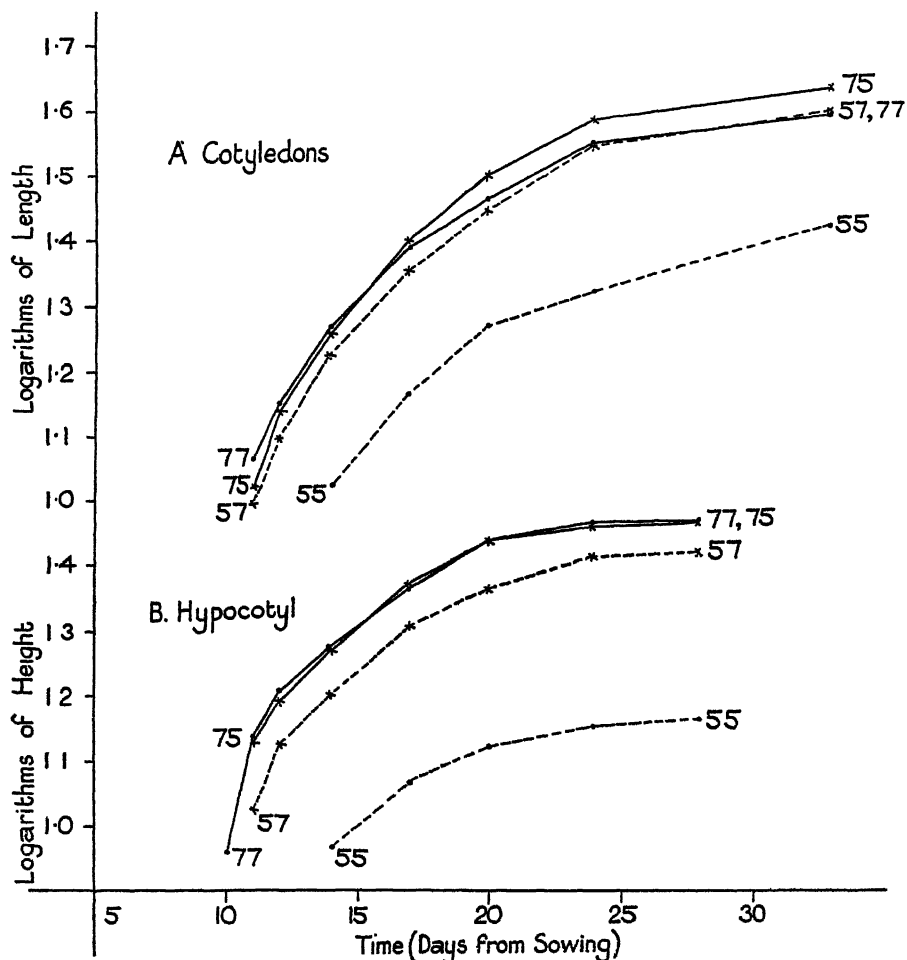


FIG. 5. Seedling growth. A. Cotyledons. Logarithms of length plotted against time. B. Hypocotyl. Logarithms of height plotted against time. Continuous lines: circles parent 77, crosses hybrid 75. Broken lines: circles parent 55, crosses hybrid 57.

occurs and complete dominance of the 77 leaf type. The first leaf of parent 55 was much smaller because of the recessive factor for dwarfness 'd'. In the 5th leaves two differences were evident; the leaf of 55 was relatively less short, while the leaves of the hybrids 57 and 75 eventually manifested size heterosis and to the same degree. In the 10th leaves these tendencies were more

pronounced, for the leaf of parent 55 was now as large as that of parent 77, and the leaves of the hybrids manifested considerable heterosis. Maternal environment, acting through embryo size, thus exerted no influence on the size of any of the leaves, while, relative to parent 77, the progressive increase

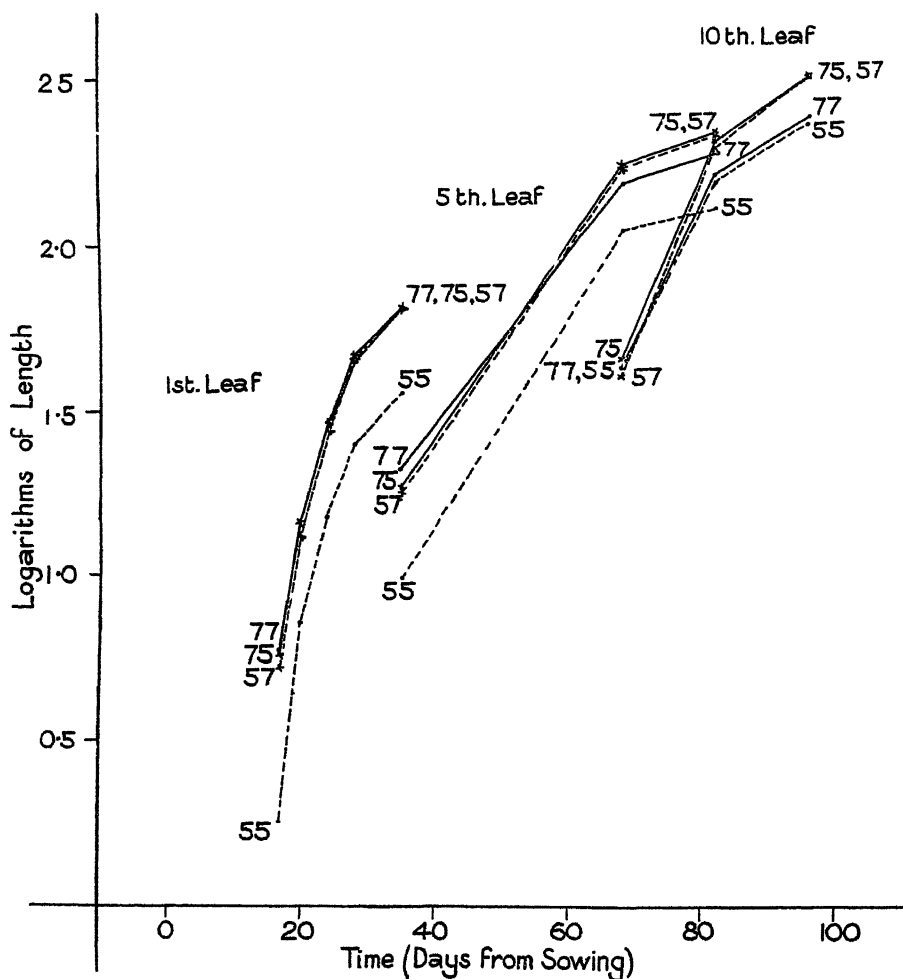


FIG. 6. Leaf development. Logarithms of length plotted against time. Continuous lines: circles parent 77, crosses hybrid 75. Broken lines: circles parent 55, crosses hybrid 57.

in size of the leaves of the hybrids 75 and 57 and parent 55 was clearly due to the same relations of fruiting that resulted in height heterosis at the 10th leaf stage.

Summing up the results of all the experiments with these genotypes, it may be said that the reciprocal hybrids 75 and 57 do not manifest heterosis in the plumule (i.e. in the region above the cotyledons) until lateral growth

and flowering begins. Embryo size has no effect at all on the growth and development of the plumular organs. Only in the cotyledons, which start development within the seed, is there any evidence of hybrid vigour during the purely vegetative phase. In this case heterosis must be attributed in general terms to increased vigour of growth. Once flowering starts this inherent vigour cannot be distinguished from the combination of favourable characters received from the two parents.

Embryo size does, however, influence the size of the cotyledons and hypocotyl. Physiologically the difference in the size of these parts may affect to a greater or lesser extent the subsequent development. Thus the hybrid 75, possessing larger cotyledons than its parent 77, should benefit from this advantage. Actually this is more than counterbalanced by the earlier germination of the latter. In this cross, therefore, as Ashby found (1937, expt. 2), there is no significant difference between hybrid and tall parent during the vegetative period.

#### DISCUSSION

Ashby (1937) in the first paper devoted to an analysis of heterosis in the tomato confined his attention to the vegetative phase prior to flowering. He realized the importance of studying also two other phases, namely, that from fertilization to embryo maturity, and the phase subsequent to flowering. The latter phase has been studied by Luckwill (1937). In the present work the former phase has received attention, though post-germination growth has also been followed.

The important role ascribed to embryo weight by Ashby and Luckwill called for a study of embryo development, for a larger embryo in the hybrid presupposes either (1) a higher relative growth rate during embryo formation, or (2) a longer period of time elapsing before dormancy supervenes. Evidence has now been presented that heterosis is displayed during embryonic development (Table VII) and a higher relative growth rate occurs in the period (20–35 days) covered by rapid development of the cotyledons. In the course of this part of the work the importance of the maternal environment became apparent. Since reciprocal hybrids were used, comparison could be made between the hybrids and the maternal homozygous parent, and it now appeared that seed and embryo size in the hybrid are determined by the maternal environment. Hybrid embryos thus belong to two phenotypes differing in weight as much as do the embryos of the homozygous parents.

An examination of variation in embryo and seed weights within a genotype showed that many factors determine these characters, of which, truss position on the plant, number of fruits in the inflorescence, and environmental factors, have been shown to be important. These are all associated with nutrition of the ripening seeds and competition between the fruits. Of the greatest importance, however, was the effect of number of fertilized ovules in the fruit,

since to a great extent this number depends upon the mode of pollination. Artificial pollination, unavoidable in securing hybrid seeds, has been shown always to be less effective so that the seed number of hybrid fruits is lower; in consequence the seed and embryo weight tend to higher levels in the hybrids than the parents so long as the latter are naturally self-fertilized. Luckwill (1939a) was aware also of the great variation in embryo and seed weight in the tomato, but his data do not distinguish between the effects of the maternal environment and of genotype, this being one of the limitations of his survey (1939) of heterosis in hybrid tomatoes.

In the light of these facts the major contribution of Ashby to the analysis of heterosis requires reconsideration. In the earlier work on maize (1930, 1932) and again on tomato (1937) the importance of embryo weight is insisted on. Hybrid vigour is successfully related to the initial advantage of embryo size in the hybrids studied. One of the hybrids used in this work was the same as that used by Ashby (1937, expt. 2), namely, the hybrid referred to throughout this paper as 57. The embryo weight as determined by Ashby (l.c., Table III) was somewhat greater than that of the parent, here called 77. It may be remarked that this is contrary to the present findings, for the hybrid with 55 as female parent would be expected to have a similar seed size and not resemble parent 77 as Ashby found. In this experiment Ashby found no heterosis, which he attributed to the equality in weight of hybrid embryo and that of one of the parents. The embryo weight found would appear to have been abnormally large, and as no precautions were taken in selecting the sample of seed the agreement in embryo weight may have been fortuitous. The paramount importance of embryo weight may thus be seriously questioned.

In the present work two samples of seed of each of the parent types were deliberately selected to secure large differences in embryo size (Table IX). The progeny of such seeds differed in none of the characters measured, showing that the small embryos must have had a higher relative growth rate and development rate. Had Ashby used reciprocal hybrids he would undoubtedly have found that embryo size is of less importance than he originally supposed.

In the light of the results stated, the size of the dormant embryo has lost its significance as an index of heterosis. In the literature also there are indications of the same conclusion. Passmore (1934), working with reciprocal crosses of *Cucurbita Pepo*, found that 'plants from small embryos have a tendency to catch up with those from large embryos', while Richey (quoted Ashby, 1937) repeating Ashby's experiments obtained 'a significantly higher growth rate of the hybrid plants for the first two weeks after germination'. In wheat hybrids Engledow and Pal (1937) found no increase in embryo size but a higher growth rate after germination. Luckwill (1939) also records that reciprocal crosses of tomato attain the same size, and Fabergé (1936), working with tetraploids, found that a 30 per cent. increase in size over diploid embryos

was lost in the seedling stage, supporting the conclusion that the size of the embryo does not determine the final size of the plants.

It was from a study of maize that Ashby formulated his physiological theory of hybrid vigour, but the results of Sprague (1936) and Bindloss (1939) suggest that here also embryo size may not be of primary importance. Sprague showed that reciprocal hybrid embryos of different size manifested hybrid vigour to the same degree, and produced evidence of a higher growth rate in the seedling stage. Bindloss, using the same material, could not find larger meristematic masses or larger cells in the hybrid embryos.

Again Whaley (1939 and 1939a) also found embryo size to be of little importance, and found further that there was no relation between the size of the plumular meristem and the presence of heterosis, thus proving for the tomato what Bindloss (1939) had shown for maize. Whaley's important contribution to the problem of hybrid vigour was, however, based on the examination of the apical meristem throughout the growth of the plants. Total meristematic volume sometimes decreased with the attainment of maturity, and this was attributed to the diversion of nutrient materials to the fruits as one possibility. Earlier in development progressive increases in the size of the determinate organs were paralleled by increases in the size of the meristems, and it will be remembered that in the hybrids 75 and 57 leaf heterosis was manifested progressively as flowering entered into the growth relations. Cell size and nuclear size in the apical meristem decreased during development, but much less rapidly in the hybrid types. With the onset of maturity, cell and nuclear size reached a minimum. The probable explanation offered was that in the hybrids the rate of intake of materials, and hence the vigour of growth, was more rapid. Such a case was investigated in barley hybrids by Gregory and Crowther (1928, 1931), who showed that the hybrid inherits greater efficiency in the use of nutrient ions, differing from the parents in this respect.

From the earlier organization of cotyledons and radicle the reciprocal hybrids (75 and 57), though both manifesting size heterosis compared with their maternal parent embryos, themselves showed inequality due to their distinct maternal environments. This reaction of embryo genotype to environment continued till the testa approached its determined dimensions, and then a limit, determined by the characteristics of the maternal parent, was imposed on embryonic growth. At germination the cotyledons completed their growth and once more manifested the differences observed in their earlier development. The plumule, still undeveloped in the mature embryo, did not exhibit any maternal effect and it is evident that maternal environment only influenced those tissues differentiated in the developing embryo while inside the seed. The results of Schlosser (1935) for tomato, and Thayer (1934) for *Cucurbita Pepo*, also show the importance of the maternal environment in early seedling growth.

Not only did the plumule in the hybrids 75 and 57 fail to manifest a

differential effect due to their particular maternal environment, but size heterosis was absent during the pre-flowering period. The vegetative appearance of the tall parent 77 was completely dominant and the hybrids were indistinguishable both from each other and from this parent.

From the beginning of the flowering period the hybrids 75 and 57 manifested size heterosis, but there is clearly a genetical explanation of this related to the fruiting characters of the plants and the relative amounts of fruiting and vegetative growth in the genotypes.

It should be pointed out that in his survey of heterosis in tomato hybrids Luckwill (1939) did not consider their fruiting relations at all, and this in light of the present work and of Whaley's data seriously detracts from the value of his survey.

Summarizing this discussion it may be said that, as Ashby surmised, the conditions of development of the reciprocal hybrid embryos play a determinate role in the final size at maturity; thus size is no measure of heterosis but of the nutritional conditions provided by the maternal parent. Nevertheless, heterosis is displayed during embryo growth, the hybrid in each case having a higher growth rate than the maternal homozygous parent. This higher growth rate appears at the time of development of the cotyledons, and does not affect the terminal meristem. Ashby's suggestion that heterosis is determined by a greater meristematic mass is thus not substantiated by this work, though Luckwill presents evidence in this direction (1937, p. 398). Since the terminal meristem is not more massive in the hybrid it is not surprising that evidence of heterosis is found only in the immediate post-germination stage while the pre-formed cotyledons and hypocotyl are completing their growth. After this, during the purely vegetative phase, heterosis does not appear, confirming Ashby's findings. The relative growth rates of all genotypes is the same; it is only with the onset of flowering and fruiting that large differences in relative growth rate reappear. This, as has been shown, depends upon a nexus of physiological processes each genetically determined. At this stage it is a combination of favourable factors as set out in Table IX inherited from the two parents which contribute to the greater growth of the hybrid. It can scarcely be doubted that the theory of Jones (1917), that of the complementary action of dominant factors, receives support.

Ashby, in his original work, did not go beyond the evidence then available. Indeed he already foresaw that embryo size may not always be the determinant factor, and the present work supports this in throwing the emphasis elsewhere. It must be admitted that the higher growth rate of the hybrid has to be ascribed to factors at present unanalysed. The mere fact that differences in relative growth rate occur within a genotype, as the progeny of seed selected for weight differences within the parent types show (Table IX), indicates that variation in relative growth rate does not necessarily indicate genetical differences. Indeed, this fact alone must throw some doubt on the whole method of investigation initiated by Ashby as a contribution to the

analysis of hybrid vigour. The work which he initiated has led to a closer study of the developmental processes in the tomato, but has not solved the problem of hybrid vigour.

#### SUMMARY

Two varieties of *Lycopersicum esculentum*, a tall commercial variety ('Blaby') with large red fruits and simple inflorescences and a dwarf variety with small yellow fruits and compound inflorescences, were investigated together with their reciprocal hybrids.

1. The factors determining embryo size and seed size were studied and the primary ones seed number ( $N$ ), seed weight ( $S$ ), and fruit weight ( $F$ ) yielded the partial correlations  $r_{NF.S} = +0.843$ ,  $r_{NS.F} = -0.638$ ,  $r_{FS.N} = +0.589$ , all highly significant. With constant seed number there is high positive correlation between fruit and seed weight. Seed and embryo size are largely determined by the number of seeds in a fruit, and thus by the effectiveness of pollination. Cross pollination by hand is less effective than natural self-pollination, leading to hybrid fruits with fewer seeds and thus larger embryos. The effect of position of fruit on the plant is considered in relation to seed size.

2. During development of the embryo, size heterosis appears at the stage of differentiation of the cotyledon primordia. At maturity the embryo size is determined by factors in the maternal embryo-sac. The genetically similar reciprocal hybrids thus constitute two phenotypes having embryo sizes the same as with that of the pure line maternal parent.

3. After germination the heterosis of the cotyledons, temporarily masked during dormancy, reappears and is reflected in their final size. The plumule shows no size heterosis during the vegetative stage of growth before flowering, and the two hybrids resemble completely the tall parent.

4. Heterosis in the hybrids becomes very evident after the onset of flowering.

5. There are no differences between reciprocal hybrids which indicate that embryo size is in any way determinate. This conclusion is supported by the uniform size attained by plants grown from seeds of the same genotype but differing considerably in weight.

6. Very considerable weight heterosis is found in the mature plants, fruit yield in the hybrids being more than twice that of the tall parent and more than three times that of the dwarf parent. This (as shown in Table IX) results from the combination in the hybrids of favourable factors inherited from the parents.

I wish to express my gratitude to Professor Macgregor Skene, in whose department these experiments were conducted, and to Professor Eric Ashby for their constant encouragement. Professor Ashby, although in Australia, has maintained great interest in the work, and has given valuable comments on the results presented in this paper. I wish also to thank Professor F. G. Gregory for his stimulating assistance in the preparation of the manuscript. To the Colston Research Society I am indebted for a grant in aid of the research.



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# Concerning the Upward Movement of Soil Solutes<sup>1</sup>

BY

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## With one Figure in the Text

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## I. INTRODUCTION

THOUGH there is now satisfactory evidence (Mason, Maskell, and Phillis, 1936; Clements and Engard, 1938; Phillis and Mason, in press) that soil solutes may move upwards through the wood alone in amounts approximating those moving in the intact stem, yet the evidence that soil solutes may also ascend in the isolated bark in approximately normal amounts is much less satisfactory. It must be emphasized that if soil solutes move through the phloem by a process analogous to diffusion, they should be able to travel upwards in the phloem, at least in stems in which the wood has been removed or in which the transpiration current has been arrested. It will be clear that in the case of solutes, such as calcium, which do not appear to be mobile in the phloem, upward movement must occur exclusively in the wood.

The evidence in favour of a movement of soil solutes through the bark is briefly as follows: Curtis (1925) showed that nitrogen might move upwards through stems from which a section of wood was removed. Mason and Maskell (1931) criticized this work on the grounds that Curtis did not show that there was any absorption of nitrogen by the roots, and that consequently he may have been merely measuring a movement of storage nitrogen. Mason, Maskell, and Phillis, using defoliated plants growing in a saturated atmosphere, analysed the whole plant and showed that while there appeared to be some upward movement of nitrogen through stems from which a section of

<sup>1</sup> Paper No. 25 from the Physiological Department of the Cotton Research Station, Trinidad.

wood had been removed, the movement was not statistically significant. On the other hand, they showed that as much nitrogen moved through ringed plants (i.e. through only the xylem) as moved through normal plants containing bark and wood.

Gustafson and Darken (1937 and 1937*a*), using radioactive phosphorus as a source of phosphorus supply to the roots and working with plants from which a section of wood had been removed, claimed to have demonstrated that radioactive phosphorus could be conducted upwards in the bark. In these experiments they made no provision for the supply of water to the plant above the cut in the wood. Later experiments (Gustafson, 1939) have shown that when water is supplied, the amount of phosphorus transported into the top is very greatly reduced. Gustafson concluded from his most recent experiments that 'there is undoubtedly some upward conduction of minerals in the phloem under normal conditions'; but his experimental results do not support this conclusion, for his plants were not *normal* inasmuch as the wood was removed. Further, it is uncertain whether they even demonstrate movement through the bark, for when he supplied water to the cut, thereby greatly reducing uptake by the top of the plant, the leaves above the cut still wilted and there may consequently have been some water movement in the bark.

Thus the position seems to be that while the isolated wood can transmit soil solutes in approximately normal amounts and therefore at normal rates up the stem, it is not yet known whether the isolated bark can do so. In the present paper an experiment designed to answer this question is described.

## II. UPWARD MOVEMENT IN THE PHLOEM OF NITROGEN AND BROMINE

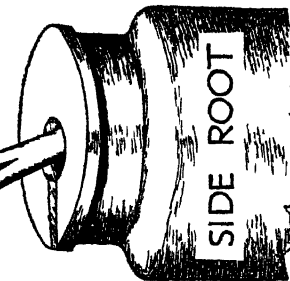
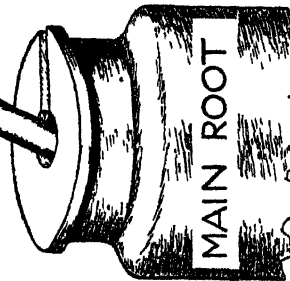
### A. *Method.*

To demonstrate upward movement of soil solutes through the isolated bark, it seems necessary to arrange for a gradient from the root to the top and also to ensure that the top is adequately supplied with water, for of course the continuity of the wood between the root and the top must be interrupted.

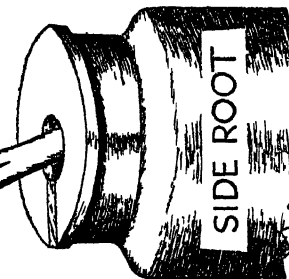
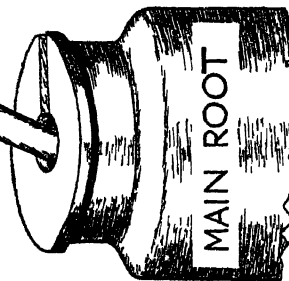
A variety of methods have been used to ensure that after a section of wood has been removed from the stem the top of the plant does not dry out. Curtis defoliated and attempted to supply water to the cut wood. Mason, Maskell, and Phillis also defoliated and maintained the plants in a saturated atmosphere. Gustafson and Darken used mostly succulent plants, and made no provision for water-supply to the tops. In the experiment described below we have used plants with double roots, one used to supply water via the wood and the other nitrogen and bromine via the bark (see page 767, 'cut-wood' group).

The plants were allowed to develop under conditions of nitrogen starvation and the concentration around the root was greatly increased at the time the experiment began. In addition to nitrogen the element bromine was employed, the plants being allowed to develop without any trace of bromine in the nutrient solution, and bromine being added to it at the same time as the concentration of nitrogen was increased. In order to steepen the nitrogen

NORMAL



CUT-WOOD



Sketch showing treatment of plants in normal and 'cut-wood' groups.

gradient along the phloem from the root to the top, the older leaves were removed from the stem, for they help to supply the younger leaves.

### B. Procedure.

Sea Island cotton plants were grown in water culture with a full mineral solution rather low in nitrogen and containing no bromine. They were approach-grafted in pairs and the weaker top removed, leaving plants with a single top and two roots, each in a separate container, as shown on page 767. The root in direct connexion with the top will be referred to as the main root and the grafted root as the side root.

When the plants were eight weeks old and had reached a height of about 60 cm., leaves were removed starting from the bottom, so that only six leaves remained on the plant. Actually about half the leaf area was removed. Nine samples each of 10 plants were then drawn. Three of these samples served as an initial collection, and, after a period of five days, three for a normal group and three for a 'cut-wood' group. The subsequent procedure was as follows:

- Day 0 (a) Nutrient solution of side root removed and, after washing with water, replaced by calcium sulphate solution.
- (b) Bark on stems of all plants between fork and roots prised away from wood over a distance of 3 in. (see page 767).
- (c) In normal plants a  $\frac{1}{2}$ -inch section of stem (bark and wood) removed between fork and side root. In 'cut-wood' plants, a  $\frac{1}{2}$ -in. ring of bark removed between fork and side root and an  $\frac{1}{8}$ -in. piece of wood removed between fork and main root.
- (d) Solution to main root replaced by one containing 400 p.p.m. each of nitrogen and bromine.
- (e) Initial collection of plants taken.
- Day 5 Collection of normal and 'cut-wood' plants.

Before collection appropriate sections of wood were removed to make groups strictly comparable.

### C. Results.

It will be clear that all nitrogen, bromine, and water entering the top must have been supplied in the normal group by the main root, and that in the 'cut-wood' group water (including calcium sulphate) must have been supplied only by the side root and nitrogen and bromine only by the main root. The results are shown in Table I. The stem between the break in the wood and the main root is included in the main root.

It will be observed that no bromine was present at the initial collection and that during the five days the experiment lasted more than three times as much bromine entered the normal as entered the 'cut-wood' group. Interrupting the continuity of the wood has thus greatly interfered with the

absorption of bromine. The weight of bromine is much the same in the main root of both groups. Considerable amounts travelled up the normal stem, but only traces, though significant ones, travelled through the bark of the 'cut-wood' group. Thus bromine seems to travel with difficulty upwards through the isolated bark.

TABLE I

*Weights of Nitrogen and Bromine in Rest of Plant, Main Root, and Whole Plant for Initial, Normal, and Cut-Wood Treatments and Changes<sup>1</sup> during the Experiment (expressed as gm. per Sample of 10 Plants)*

	Rest of Plant.		Main Root.		Whole Plant.	
	Nitrogen.	Bromine.	Nitrogen.	Bromine.	Nitrogen.	Bromine.
Initial . . .	1.891	0.000	0.416	0.000	2.307	0.000
Normal . . .	2.378	0.169	0.416	0.052	2.794	0.221
Cut-wood . . .	2.034	0.012	0.454	0.054	2.488	0.066
Normal—initial . .	0.487	0.169	0.000	0.052	0.487	0.221
Cut-wood—initial .	0.143	0.012	0.038	0.054	0.181	0.066

As nothing is known concerning the (downward) mobility of bromine in the phloem, its use in experiments of this nature is not perhaps warranted. Cotton, it should be added, seems to tolerate very high concentrations of bromine (6,000 p.p.m.) without suffering sensible injury.

The uptake of nitrogen was also greatly diminished by cutting the wood. The uptake of nitrogen by the 'cut-wood' group during the five days the experiment lasted was only 0.181 gm. as compared with 0.487 by the normal group<sup>2</sup>. All the nitrogen absorbed by the normal group travelled to the 'rest of plant', while more than 75 per cent. of that absorbed by the 'cut-wood' group reached the 'rest of plant'. It thus seems clear that the isolated bark may transmit nearly the same proportion of the nitrogen absorbed by the root as is transmitted through the intact stem. It is, of course, quite impossible to say how much of the nitrogen ascending the stem of the normal group travelled in the bark and how much in the wood. Any injury suffered by the bark of the 'cut-wood' group would tend to reduce the amount travelling in the bark of this group below that travelling in the bark of the normal group, while on the other hand the gradient in the bark of the 'cut-wood' group ought to have been steeper than in the bark of the normal group, and this would tend to increase movement in the bark of the 'cut-wood' group over that in the normal group.

<sup>1</sup> Fully significant ( $P = 0.05$ ) changes are italicized.

<sup>2</sup> Nitrogen was supplied as ammonium nitrate. Determinations of nitrate nitrogen and ammonia nitrogen showed that in the root these forms could only account for 0.03 gm. out of the total nitrogen uptake of 0.181 gm. It thus seems clear that there was no accumulation of inorganic nitrogen in the root, and consequently that we are not dealing with a replacement of storage nitrogen by inorganic nitrogen absorbed by the root.

## III. DISCUSSION

It seems clear that nitrogen absorbed by the root may be transmitted upwards in the bark at rates approximating those at which it travels in the intact plant, i.e. bark plus wood. It has also been demonstrated, as stressed in the introduction, that the wood alone can transmit upwards the nitrogen absorbed by the root at rates comparable with those at which nitrogen moves upwards in the intact plant. Which channel is utilized and to what extent in the normal plant is not clear, for it will be evident that all experiments carried out on plants from which one channel has been removed are inadmissible.

The presence of salts in the transpiration current would certainly suggest that this channel plays at least some part in the upward transmission of soil solutes. An increase in the amount of any one or all of the mineral elements in the leaf during the day and a loss during the night<sup>1</sup> would also suggest upward movement in the wood and downward movement in the bark. *A priori* considerations suggest that elements (e.g. potassium) that do not undergo chemical transformation might accumulate in the leaf while transpiration was in progress and that a positive gradient from the leaf to the root would be generated, which would stop upward movement in the bark. For an element that is transformed wholly in the leaf there might be upward movement of the inorganic fractions in both bark and wood and downward movement of the organic fraction in the bark. For elements like calcium that are immobile in the phloem all upward movement would of course occur in the wood.

## IV. SUMMARY

1. An experiment is described in which the uptake of nitrogen and bromine by the root and their subsequent distribution throughout the plant in normal plants is compared with that in plants in which the continuity of the wood was broken at the base of the stem.
2. The supply of water to the foliage region of the 'cut-wood' plant was ensured by a side root, grafted into the stem above the break in the wood.
3. The uptake of nitrogen and bromine by the roots was greatly reduced as a result of cutting the wood.
4. All the nitrogen absorbed by the root of the normal plant travelled into the foliage region, while 79 per cent. of that absorbed by the 'cut-wood' roots did so.
5. 76.5 per cent. of the bromine absorbed by the normal plant travelled to the foliage region, while only 18 per cent. of that absorbed by the 'cut-wood' plant did so.

<sup>1</sup> Evidence of this state of affairs can only be accepted if the results are expressed on the sample basis and show statistically significant changes. Our own experiments (unpublished) prove that there is an increase in the potassium content of the leaf during the day and a loss to the rest of the plant during the night.

6. It is concluded, at least in the case of nitrogen, that this element may travel upwards in the bark at rates comparable with those which occur in the intact stem.

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# Studies on the Partition of the Mineral Elements in the Cotton Plant

## II. Preliminary Observations on Potassium, Calcium, and Magnesium<sup>1</sup>

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With six Figures in the Text

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## I. INTRODUCTION

IN the first paper of this series (Phillis and Mason, 1939) we attempted, admittedly in a very tentative manner, to distinguish in the leaf between those forms of nitrogen and phosphorus that are especially concerned with growth (increase in dry weight) and those forms that are 'luxury consumed.' The underlying idea was that when the tissues are rich in these elements the luxury forms might be relatively abundant and that when the tissues are poor the growth forms would be relatively abundant. The degree of saturation of

<sup>1</sup> Paper No. 24 from the Physiological Department of the Cotton Research Station, Trinidad.

the tissues was judged by the amount of nitrogen and of phosphorus per 100 gm. dry weight, and the relative abundance of the growth forms by the partition indices, i.e. for nitrogen, the weight of protein nitrogen as a percentage of total nitrogen, and for phosphorus, the weight of insoluble phosphorus as a percentage of total phosphorus.

It was found in three experiments that over wide ranges of nitrogen, phosphorus, and potassium supplies the partition index for nitrogen was negatively correlated with the weight of nitrogen per 100 gm. dry weight. This was also the case for phosphorus under varying nitrogen and phosphorus supplies, but at high levels of potassium supply the correlation coefficient between phosphorus per 100 gm. dry weight and the partition index for phosphorus tended to be positive. The general conclusion was drawn that luxury nitrogen tended to accumulate as some form of organic crystalloid nitrogen and also as nitrate nitrogen, and that under varying nitrogen and phosphorus supplies luxury phosphorus tended to accumulate as phosphate phosphorus. In the present paper we have attempted to apply the same method to potassium, calcium, and magnesium.

## II. METHODS

In a recent paper (Mason and Phillis, 1936) we pointed out that there were some grounds for the belief that an appreciable part of the potassium of the leaf was not in solution in the sap. It was suggested that this insoluble fraction might be adsorbed. The method used for estimating the partition of potassium was briefly as follows: The concentration was determined in sap expressed from leaves which had been killed by freezing at about  $-15^{\circ}\text{C}$ . From the sap concentration and the weight of free water in the sample, the weight of sap-soluble potassium was calculated. The total weight of potassium in the sample was determined on dried material and on the hot-water<sup>1</sup> extract from the fresh material. It is immaterial which method is used as both give the same value for total potassium. The weight of adsorbed potassium was taken to be the difference between the total weight in the sample and the weight of the sap-soluble fraction. The value of the adsorbed fraction expressed as a percentage of the total potassium is referred to as the partition index for potassium.

It was found that this boiling-water extraction process, which removed all the potassium and also all the chlorine, did not remove all the calcium and magnesium. The weight of these elements determined on the dried material always exceeded that determined in the extract with boiling water. Thus, for these two elements there is in addition to the sap-soluble and adsorbed

<sup>1</sup> 50 gm. samples of leaf material were covered with 300 ml. of boiling water. They were allowed to stand overnight. Next day, the water extract was decanted and replaced by the same volume of boiling water. After standing overnight, this procedure was repeated. In all, five such extractions were made and the total volume of extracts and washings were made up to 2 litres. Aliquots were taken from this for analysis.

fractions a *combined* fraction. The nature of this combined calcium has been investigated by Kostytschew and Berg (1929). Their results will, however, be considered more conveniently in the discussion. In the case of magnesium very little appears to be known about the combined fraction. The magnesium present in the chlorophyll would not account for more than about 0.03 gm. per 100 gm. dry matter (chlorophyll content of dry matter reckoned as 1.2 per cent. (Spoehr, 1926)), whereas values for combined magnesium as high as 0.22 gm. per 100 gm. dry matter are recorded in the present paper.

The combined fractions for calcium and magnesium have been found to be surprisingly little affected either by the potassium supply, by the richness of the tissues in calcium and magnesium respectively, or by the age of the leaf. In calculating the partition indices for calcium and magnesium we have therefore omitted this relatively inert fraction and for these elements, as for potassium, the partition index represents the adsorbed (i.e. boiling-water extract minus sap-soluble) fraction expressed as a percentage of the sum of the sap-soluble and adsorbed fractions (i.e. boiling-water extract).

### III. DOES ADSORPTION OCCUR AFTER DEATH? (Experiments 1 and 2)

Whether the adsorbed fractions exist as such in the living plant or whether adsorption takes place after death will now be considered. In our original paper suggesting the presence of adsorbed potassium we said 'if it could be demonstrated that the solute composition of the sap was independent of the method of destroying permeability, there would be grounds for the belief that the expressed sap was a representative sample of the sap in the tissue'. We compared the concentrations of potassium, calcium, magnesium, and chlorine in sap extracted from leaves which had been killed by freezing with the concentrations found in sap extracted from leaves killed by exposure to toluene vapour. It was found that the differences for potassium, magnesium, and chlorine were small and not significant, but that the concentration of calcium was significantly less in the toluene treatment.

#### A. *Experiment 1*

In further experiments to test this point we have compared the concentrations in sap expressed from leaves killed by freezing with those in sap expressed from leaves killed by exposure to toluene vapour and to heat. Samples were killed by exposure to  $-15^{\circ}\text{C}$ . for sixteen hours, by exposure for four hours to an atmosphere saturated with toluene vapour, and by exposure to  $100^{\circ}\text{C}$ . for thirty minutes. The leaves killed by cold and by heat were packed in test-tubes before immersion in the ice-salt mixture and in boiling water respectively. The saps were then expressed. Three samples were used for each treatment. The mean results for each treatment are shown in Table I.

TABLE I

*Effect on Sap Concentrations of Exposure of Leaves to 100°C, to Toluene Vapour, and to -15°C*

	Mg. per 100 gm. water				Mg. equivalents per 100 gm. water.
	Cal- cium.	Magne- sium.	Potas- sium.	Chlo- rine.	Total bases.
Heat . . . . .	531.5	67.9	678.5	470.6	49.6
Toluene vapour . . . . .	550.5	66.2	652.6	479.8	49.8
Cold . . . . .	568.2	62.9	656.2	476.6	50.5
S.D. { $P = 0.05$ . . . . .	5.7	1.8	17.9	17.6	0.7
{ $P = 0.10$ . . . . .	4.5	1.4	14.2	14.0	0.6
Maximum percentage change . . . . .	6.9	8.0	4.0	2.0	-

It will be seen that the concentration of chlorine was not much (2 per cent.) affected by the different treatments. This may be taken as evidence that the moisture content of the tissues was not greatly affected. For potassium the concentration in the sap expressed from the boiled leaves was significantly greater than those from the 'toluened' and frozen leaves. For magnesium the concentration for the frozen leaves was significantly less than those from the 'toluened' and boiled leaves. Calcium shows the same behaviour as in our previous paper. The concentration in the toluened leaves was less than that from frozen leaves; again the difference is fully significant. In the case of the boiled leaves the concentration is less than in the toluened and the frozen leaves. Thus boiling as compared with freezing leads to a reduction in the concentration of calcium and to an increased concentration of magnesium and potassium in the sap. It will be noticed that the concentration of total base equivalents is nearly the same for the boiled and frozen leaves. The actual difference amounts to only 1.8 per cent., but is in fact fully significant. It is nearly the same as the difference in chlorine concentration.

### B. Experiment 2

In order to obtain more information concerning the differences in concentration between sap expressed from boiled and frozen leaves, the following experiment was carried out. Three samples of leaves were frozen in the usual way and the sap expressed. Another 12 samples were packed in test-tubes and immersed in boiling water. Triplicate samples were withdrawn after periods of twenty minutes, four, eight, and twelve hours, and the sap was expressed from these samples immediately after cooling. The results (mean of 3 samples) are shown in Table II. As there was some loss (about 10 per cent.) of water during boiling, the results are expressed as the weight per 100 gm. original (i.e. before boiling) fresh weight. Results are available for calcium and potassium but not for magnesium.

TABLE II

*Effect of Freezing and Heating to 100°C on the Partition of Potassium and Calcium*

	Potassium (mg. per 100 gm. original fresh weight)			Parti- tion Index.	Calcium (mg. per 100 gm. original fresh weight)				Parti- tion Index.
	Total.	Solu- ble.	Ad- sorbed.		Total.	Solu- ble.	Com- bined.	Ad- sorbed.	
Frozen . . . . .	396	341	55	13.9	585	328	219	38	10.4
Heated (20 min.) . . .	386	348	38	9.8	591	302	244	45	13.0
„ (4 hours) . . . . .	392	362	30	7.7	601	272	247	82	23.2
„ (8 „) . . . . .	397	377	20	5.0	587	283	232	72	20.3
„ (12 „) . . . . .	394	367	27	6.9	595	277	224	94	25.3
S.D. { $P = 0.05$ . . . . .	9	28	23	6.0	38	28	45	24	5.4
$P = 0.10$ . . . . .	7	23	19	4.9	30	22	36	19	4.3

It will be seen that the effect of exposure to a temperature of 100° C. has been to increase the amount of soluble and to decrease the amount of adsorbed potassium. The partition index for potassium amounted to 13.9 in the frozen leaves and was reduced to 6.9 after twelve hours' exposure to boiling water. For calcium the boiling treatment had the reverse effect. The partition index amounted to 10.4 in the frozen leaves and was increased to 25.3 after twelve hours' exposure to boiling water. Combined calcium does not appear to have been significantly changed.

To sum up, the differences in the concentrations of potassium and calcium between sap expressed from frozen and from boiled leaves appear to be due to the occurrence of base exchange during boiling. It would seem, therefore, that the adsorbed fraction may exist as such in the living plant.

## IV. CHANGES IN PARTITION (Experiments 3 and 4)

## A. Procedure

Two experiments are described in this section. Cultural and pruning details were the same in both. Sea Island cotton plants were grown in sand cultures under glass and supplied with nutrient solutions of varying potassium concentration. In both experiments there were five levels of potassium supply and for each level approximately 70 plants. The concentration of bases in the culture solution used is shown in Table III. The plants were pruned so that they consisted only of the main axis and its leaves. When they were approximately ten weeks old the leaves were divided into three regions, top, middle, and bottom, the youngest leaves being ignored, so that each region

TABLE III

*Concentrations (p.p.m.) of Bases in Nutrient Solution used*

Potassium.	Calcium.	Magnesium.	Sodium.
12.5	120	50	205
25.0	„	„	201
50.0	„	„	192
100.0	„	„	175
200.0	„	„	140

contained an approximately uniform weight of material. In the first experiment the bark on the stem above the cotyledons which could be readily separated from the wood was divided into two equal portions referred to as Top and Middle bark, while the bark between the cotyledons and the root constituted the Bottom bark.

## B. Results

### (a) Dry weight of whole plant.

The dry weights of the whole plant in experiments 3 and 4 are shown in Fig. 1, expressed as percentages of the lowest value. It will be seen that the dry weight in experiment 3 increased up to the highest level of potassium supply, but that in experiment 4 there was no further increase after the 50 p.p.m. level of potassium supply, while the dry weight actually diminished between 100 and 200 p.p.m. The reason for this difference is not clear.

### (b) Potassium.

The results for potassium in the three regions of leaves (lamina only) are shown in the centre (experiment 3) and on the right (experiment 4) of Fig. 2. The results for the three regions of bark (experiment 3) are shown on the left of the figure.

The results for the leaves are very similar for the two experiments. In both potassium per 100 gm. dry weight increased as the supply of potassium to the roots was increased. The actual values are very similar in the two experiments. The weight of potassium per 100 gm. dry weight was greatest in the top leaves and least in the bottom leaves, except at the highest levels of supply where the top leaves were poorer in potassium than the bottom and middle leaves. It would appear that *at high levels of potassium supply the potassium gradient in the leaves is reversed, a fact which, if confirmed by field experiments, might prove of value in diagnosing the potassium status of the plant.*

The partition indices for potassium are shown in the lower part of the figure. It will be noted that the average level of these indices is higher in the bark than in the leaf. In the leaves levels are generally highest in the top and lowest in the bottom. It will be seen that in experiment 4 there was a *complete absence of adsorbed potassium in the bottom leaves at the 12.5 and 25.0 p.p.m. levels of supply.* It will also be seen that as the supply of potassium increased the partition indices tended to rise. Thus, the partition indices and the weights of potassium per 100 gm. dry weight are positively correlated. The correlation coefficients are shown in Table IV. Fully significant ( $P = 0.05$ ) correlations are shown in heavy type and partially significant ( $P = 0.10$ ) correlations in italics. It may be concluded that there is a tendency for the proportion of adsorbed potassium to be higher at high than at low levels of supply. This might suggest (see Introduction) that adsorbed potassium is especially in-

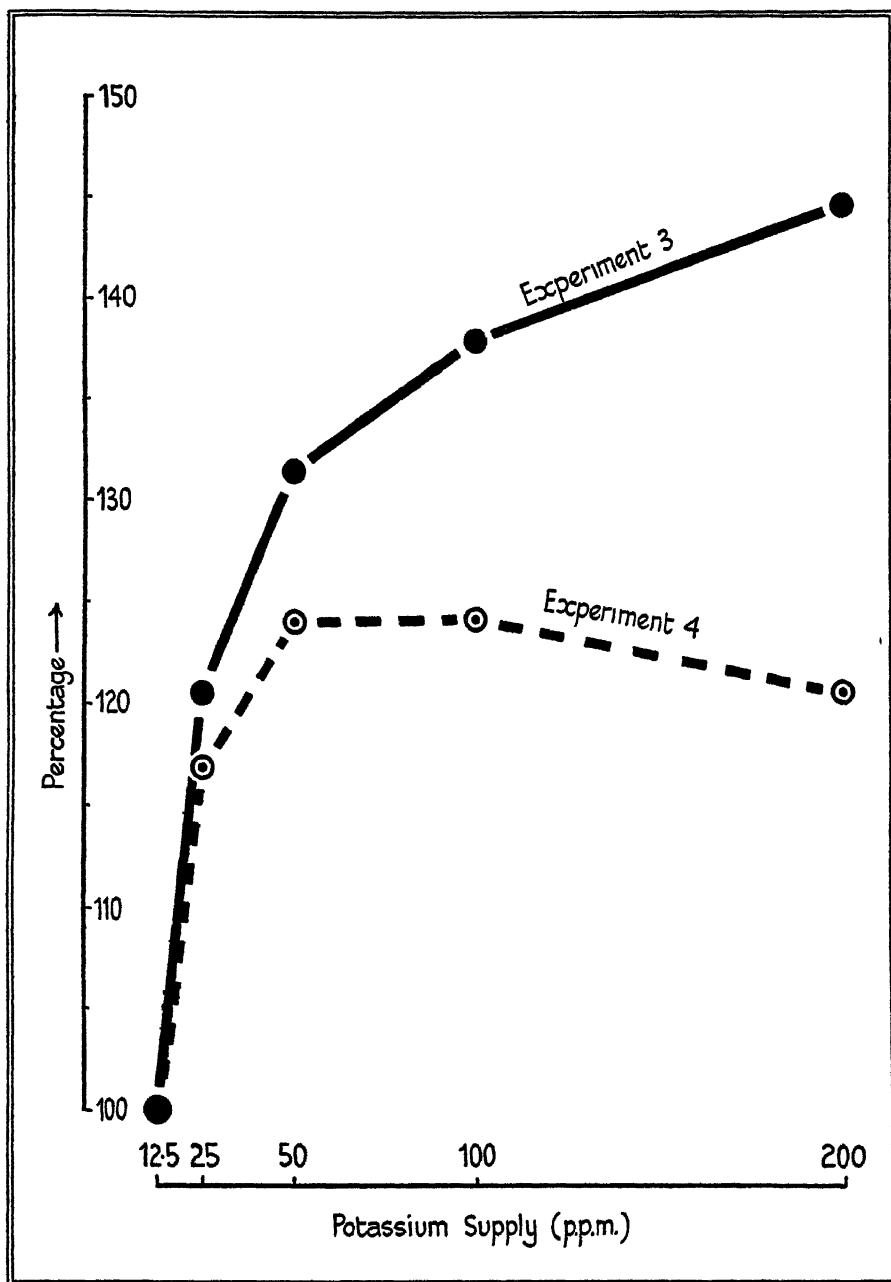


FIG. 1. Dry weights of whole plant in experiments 3 and 4, expressed as percentages of the lowest value.



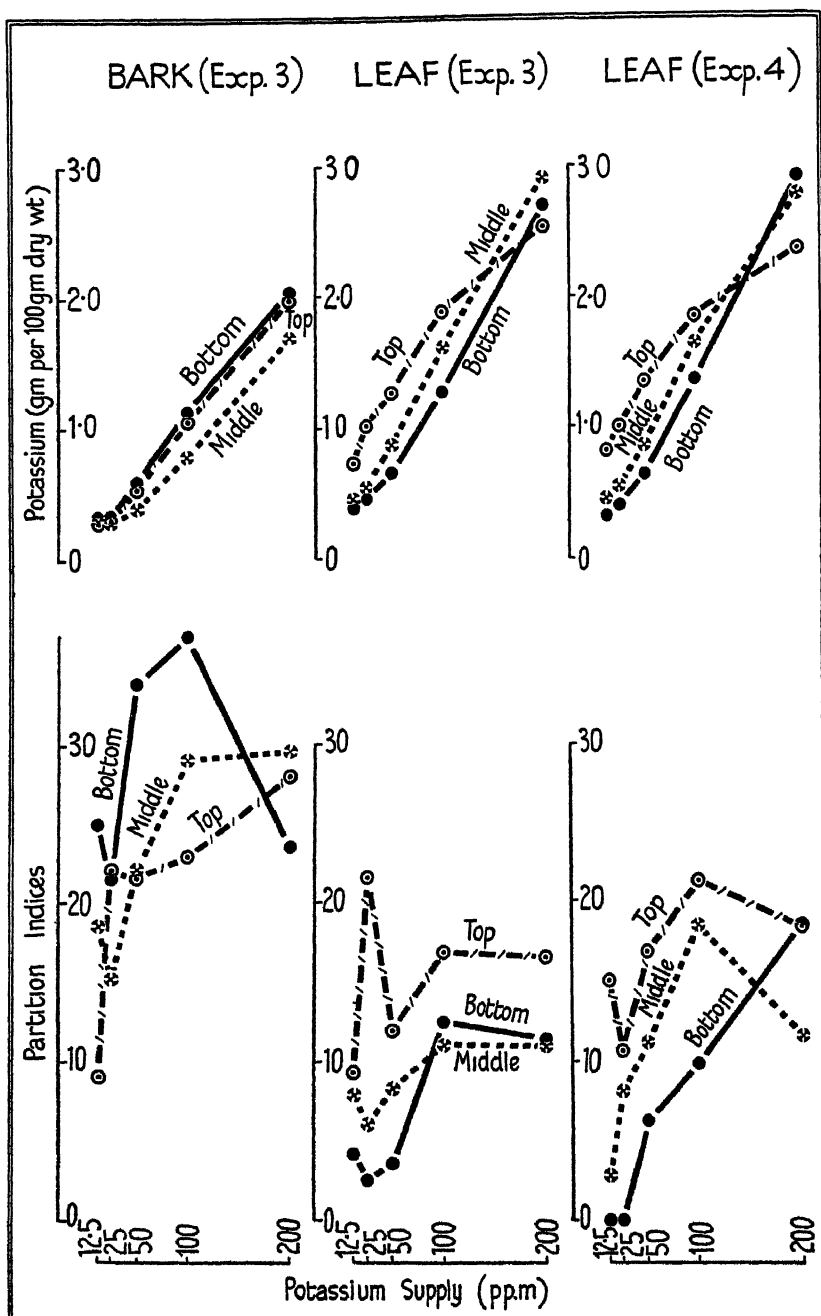


FIG. 2. Showing (above) the weights of potassium per 100 gm. dry weight and (below) the gross partition indices in bark and regions of leaves.

volved in luxury consumption and that the sap-soluble fraction is more concerned with growth. This, we shall show, is probably not the case.

TABLE IV

*Correlation Coefficients between Partition Indices for Potassium and Weights of Potassium per 100 gm. Dry Weight in Leaves (Experiments 3 and 4) and Bark (Experiment 3)*

	Experiment 3.		Experiment 4.
	Bark.	Leaves.	Leaves.
Top . . .	+0.717	+0.295	+0.642
Middle . . .	+0.837	+0.826	+0.556
Bottom . . .	+0.035	+0.789	+0.968

In the bark the weight of potassium per 100 gm. dry weight tends to be highest in the bottom region. This is the reverse of what occurred in the leaf. The partition indices are larger than in the leaf, but show the same tendency to increase as the supply to the roots increased. They are also, like those of the leaf, positively correlated (see Table IV) with the weights of potassium per 100 gm. dry weight. The only correlation to reach even the level of partial significance, however, is that for the middle region.

(c) *Calcium and magnesium.*

1. *Total calcium and magnesium.* The changes in the weights of magnesium and calcium per 100 gm. dry weight for the leaves only are shown in Fig. 3. The values for magnesium (experiment 3) are shown on the left and for calcium in the centre (experiment 3) and on the right (experiment 4) of the figure. The results for both elements differ from those of potassium in that the leaves at the bottom of the stem are richer than those at the top. The phloem-mobile element, potassium, behaves like the phloem mobile elements nitrogen (Maskell and Mason, 1929) and phosphorus (Mason and Maskell, 1931), while magnesium, which is believed (Maskell, Phillis, and Mason, in preparation) to be phloem mobile, behaves in this respect like calcium (Mason and Maskell, 1931), which is not apparently mobile in the phloem. The cause of this anomaly is not clear. The results for magnesium differ from those for calcium in that the curves for the three regions of leaves converge sharply as the supply of potassium increased. That is to say, they converge as the tissues become poorer in magnesium, for the amounts of both calcium and magnesium diminished as the amount of potassium increased. In the case of a phloem-mobile element, the element is probably entering the leaf via the wood and being re-exported via the phloem throughout development. If the rate of entry diminishes or the rate of export increases the leaf will grow

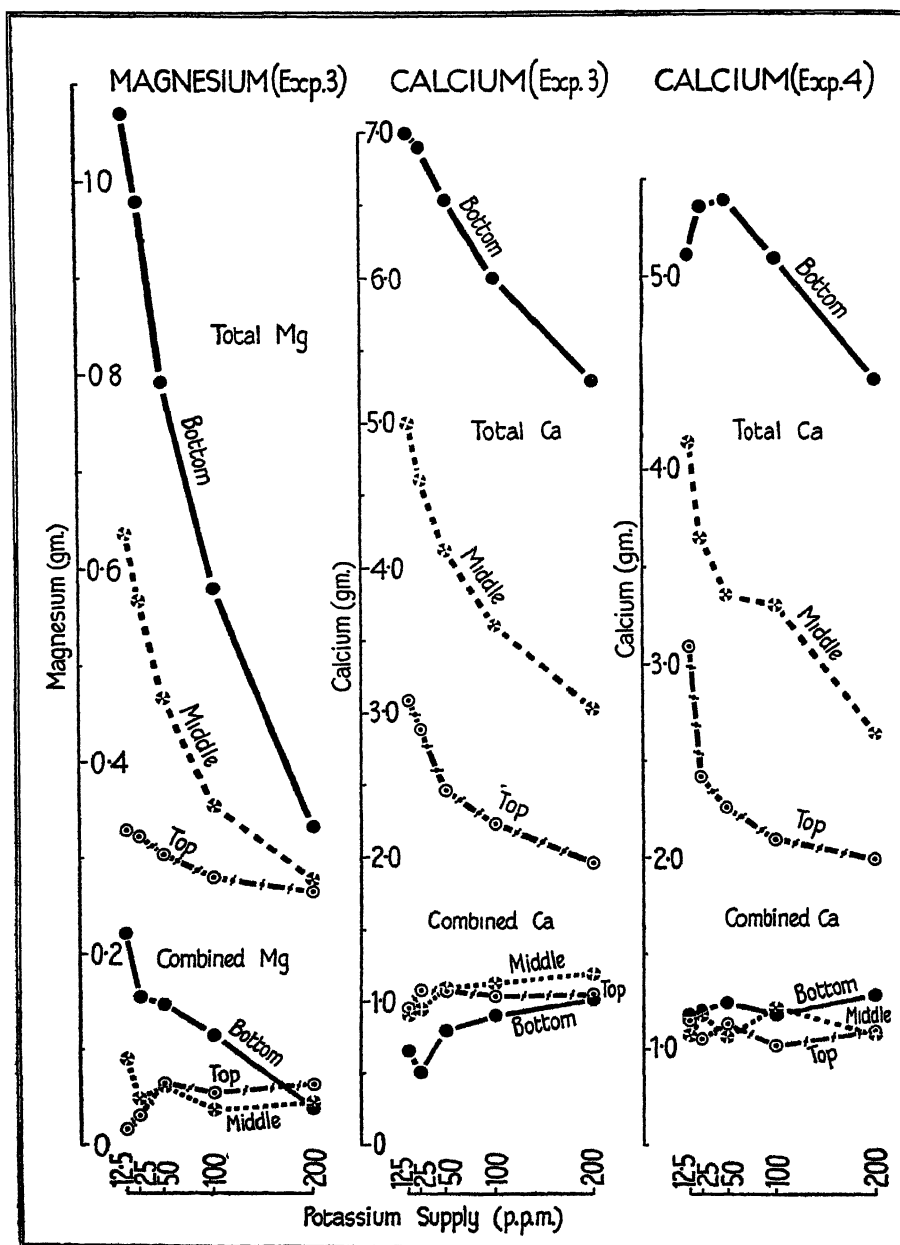


FIG. 3. Showing the weights of total calcium and magnesium (above) and of combined calcium and magnesium (below) per 100 gm. dry weight in the leaves.

poorer as it ages. In the case of an element like calcium it will accumulate throughout development. In the case of magnesium, which is apparently mobile under certain conditions, the reason for the convergence at low levels of magnesium per 100 gm. dry weight is possibly due to a relatively greater rate of export from the leaves at low than at high levels of saturation. In other words, the demand for magnesium by the non-foliar tissues may be relatively greater under starvation than under conditions of plenty.

Calcium and magnesium have each been fractionated into two sap-insoluble fractions, combined and adsorbed, and one sap-soluble fraction.

2. *Combined calcium and magnesium.* The weights of combined calcium and magnesium per 100 gm. dry weight are shown in the lower part of Fig. 3. They should be compared with the total weights of these elements per 100 gm. dry weight which are shown in the upper part of the same figure. The constancy of the combined calcium values in the two experiments will be noticed. They are not affected, except in the bottom leaves of experiment 3, by the supply of potassium or by the age of the leaf. The behaviour of combined calcium is thus quite different to that of total calcium. It will be noticed that combined calcium in the bottom leaves (experiment 3) behaves in a reverse way to that of combined magnesium. The magnesium values too are very constant except for the bottom region.

It would thus appear that the poorer the leaves are in calcium the greater is the proportion present in the combined form. This, coupled with the remarkable constancy of combined calcium per 100 gm. dry weight, might suggest that combined calcium plays an important part in dry weight production. As we shall refer to this matter again, we will pass on to consider the behaviour of non-combined calcium and magnesium.

3. *Non-combined calcium and magnesium.* The results for non-combined calcium and magnesium are shown in Fig. 4. Non-combined calcium and magnesium are comparable to total potassium in that they consist of a fraction in solution in the expressed sap and another fraction that can be extracted with boiling water. The latter fraction we have termed adsorbed. It will be seen that non-combined calcium and magnesium per 100 gm. dry weight behave in much the same way as total calcium and magnesium.

The partition indices for calcium and magnesium are shown in the lower part of the figure. In the case of magnesium, none of the correlation coefficients (Table V) between the partition indices and the weights of non-combined magnesium per 100 gm. dry weight are significant, nor is there any regularity in their behaviour. For calcium the correlation coefficients are all positive in experiment 3, and those for the middle and bottom regions are partially significant. Thus non-combined calcium behaved like potassium; the partition index is positively correlated with the weight of the (non-combined) element per 100 gm. dry weight. Under conditions of starvation the proportion of the sap-soluble calcium is high and under conditions of plenty it is low.

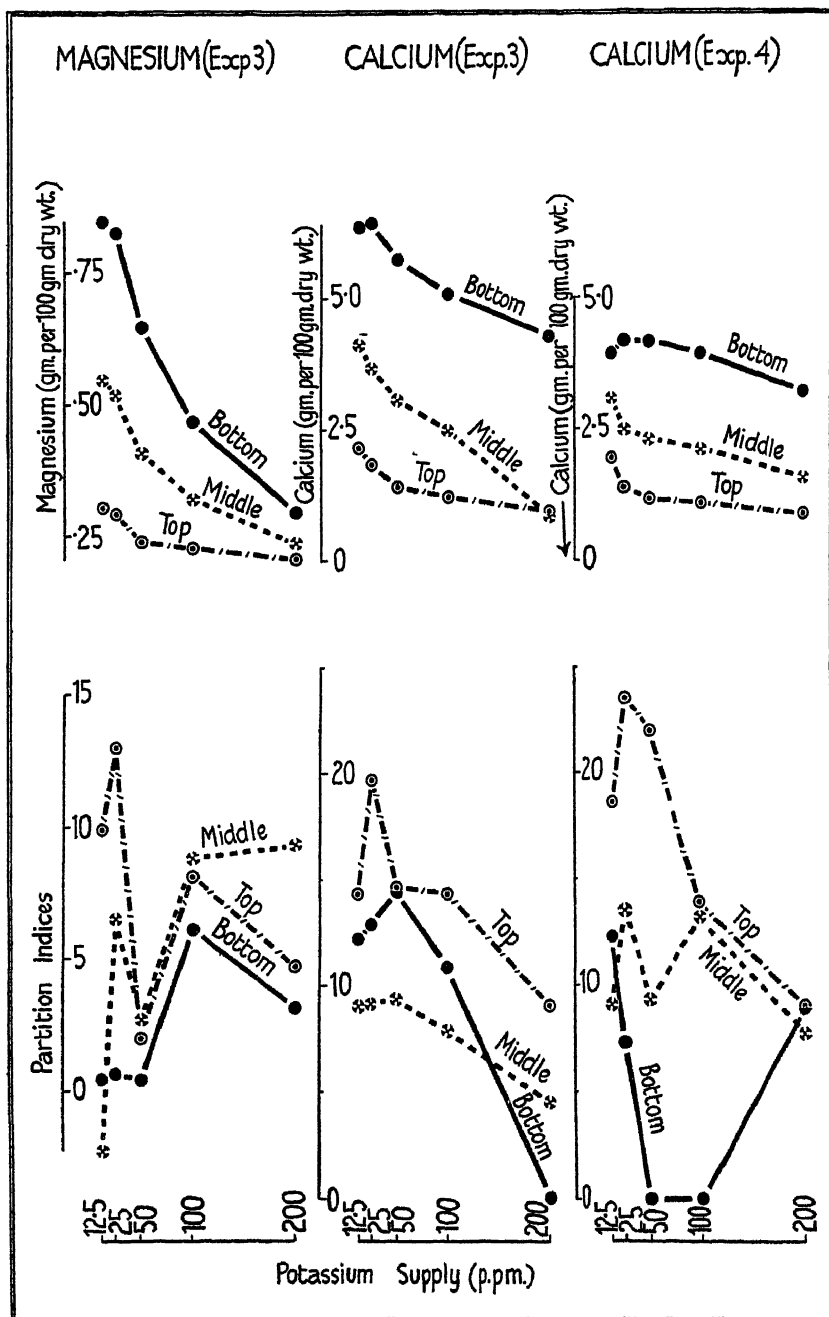


FIG. 4. Showing the weights of non-combined calcium and magnesium per 100 gm. dry weight (above) and the partition indices (below) for three regions of leaves.

TABLE V

*Correlation Coefficients between the Partition Indices for Calcium and Magnesium and the Weights of Non-combined Calcium and Magnesium per 100 gm. Dry Weight in Leaves of Three Regions (Experiments 3 and 4)*

	Experiment 3.		Experiment 4.
	Magne- sium.	Calcium.	Calcium.
Top . . .	+0.76	+0.64	+0.46
Middle . . .	-0.76	+0.83	+0.29
Bottom . . .	-0.32	+0.84	-0.38

#### V. BASE EXCHANGE (Experiment 3)

In experiment 3 we have complete analyses of calcium, magnesium, and potassium, but not of sodium. Sodium like magnesium, however, is relatively unimportant compared with calcium and potassium. Thus in experiment 3, where sodium was determined only on the leaf-sap, the mean concentration of sodium at the 12.5 p.p.m. level of supply was 47 mg. per 100 ml. sap, while the potassium concentration was 106 mg., the calcium concentration 680 mg., and the magnesium concentration 102 mg. Increasing potassium supply caused a drop in sodium concentration. At the lowest level of potassium supply (12.5 p.p.m.), it should be noted, the sodium supply was 200 p.p.m., or sixteen times as great as the potassium supply, yet the sodium sap-concentration in the leaf was less than one-half of that of potassium. The partition index for the summated bases, calcium and magnesium (omitting the combined fractions), and potassium has been calculated as follows: The soluble and adsorbed fractions of the elements are expressed in terms of gram equivalents. The sum of the adsorbed fractions is then expressed as a percentage of the sum of the corresponding values for the adsorbed plus sap-soluble fractions to give a summated base partition-index. The omission of sodium introduces only a small error which should not affect the results. These partition indices are shown for experiment 3 in Fig. 5. It will be observed that both the top and middle regions show a striking constancy over the range of potassium supply employed, while the bottom region is rather constant except at the 200 p.p.m. level of potassium supply.

To emphasize the constancy of the summated base partition index we show for the middle region the partition indices for the summated and individual elements in Fig. 6. The individual partition indices are obtained by expressing the adsorbed fraction of each element (expressed as gram equivalents) as a percentage of the total weight of non-combined calcium, magnesium, or potassium. It will be observed how calcium and potassium compensate one another; the results for magnesium hardly affect the issue. The implication is that the partition of the bases between adsorbed and sap-soluble fractions is determined by base exchange.

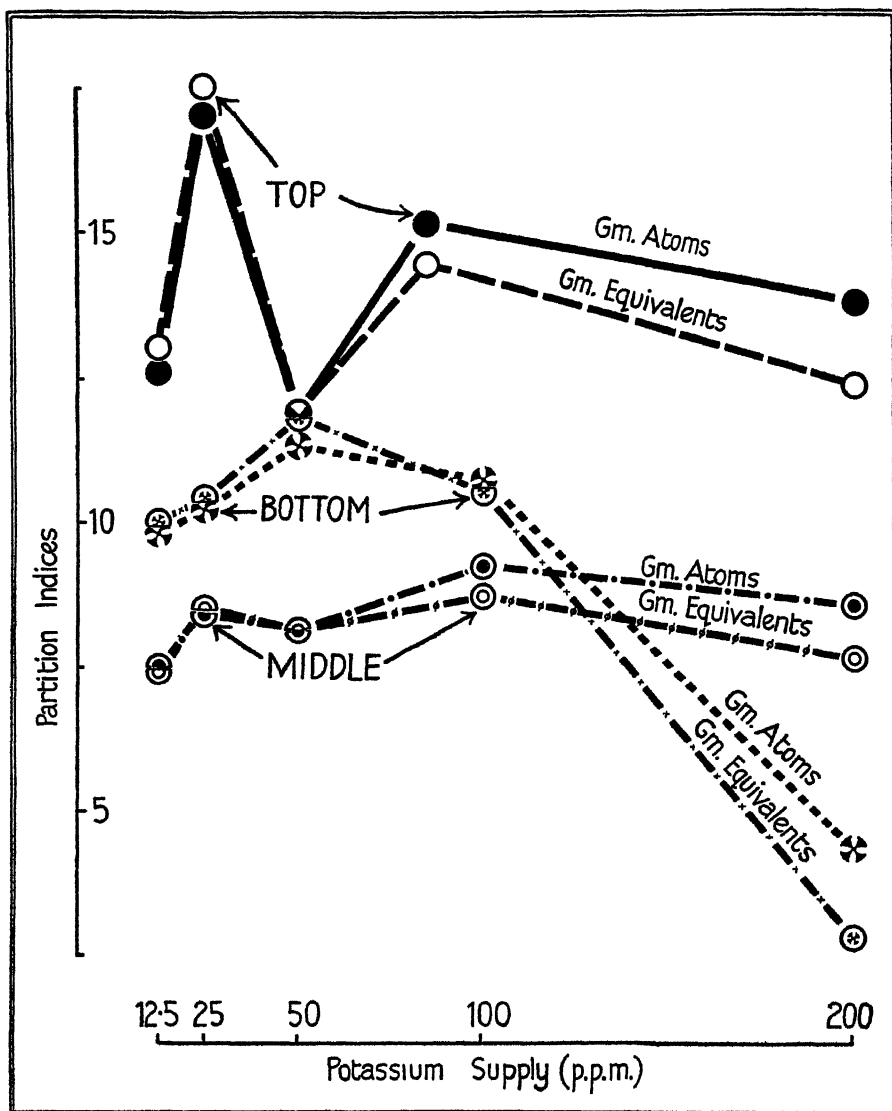


FIG. 5. Partition indices for the sum of calcium, magnesium, and potassium expressed as gram atoms and gram equivalents for three regions of leaves (experiment 3). Chemical calcium and magnesium are not included.

## V. DISCUSSION

In the introduction we pointed out that the idea underlying these investigations on partition was that when a tissue was rich in an element (measured by the degree of saturation of dry matter) luxury forms of these elements might be expected to be relatively abundant, while under conditions of starvation

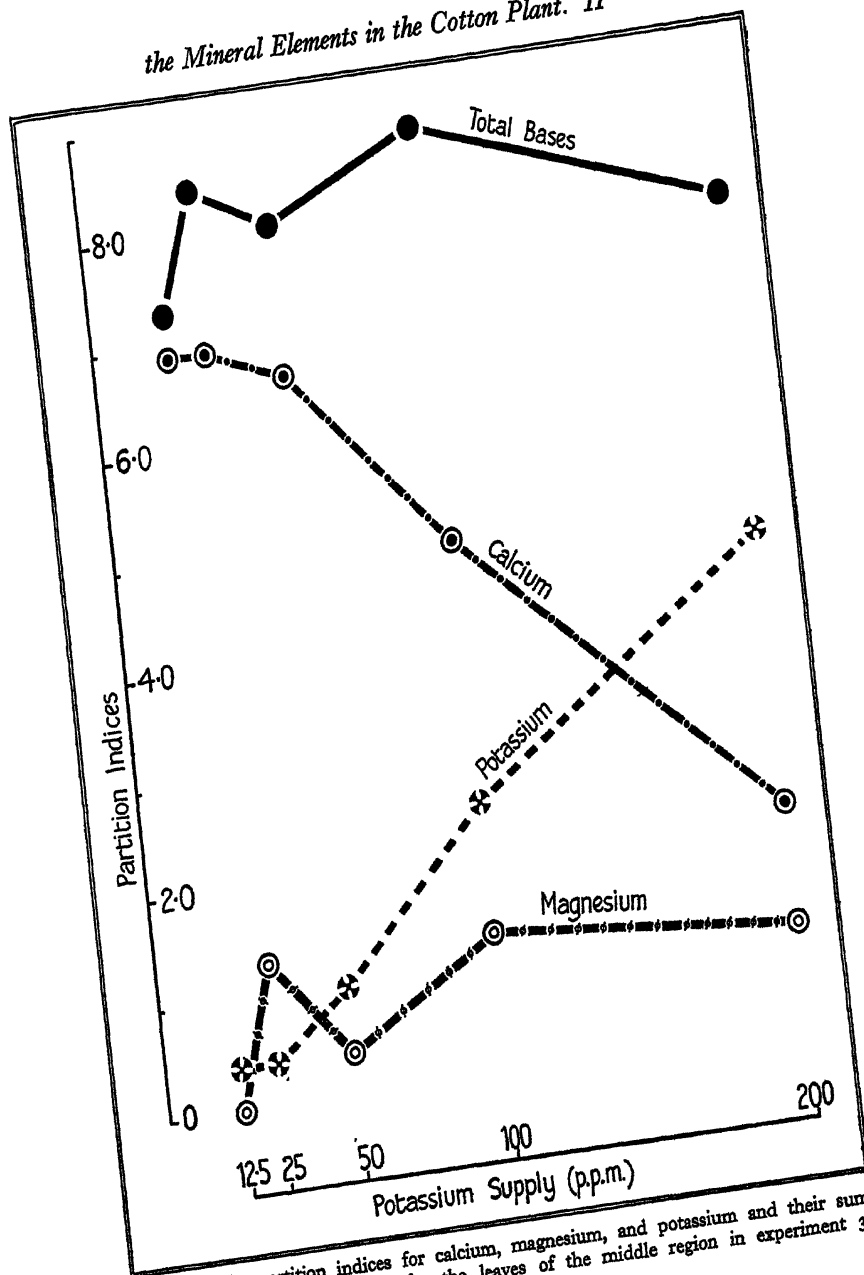


Fig. 6. The partition indices for calcium, magnesium, and potassium and their sum expressed as gram equivalents for the leaves of the middle region in experiment 3. Chemical calcium and magnesium are not included.



the forms of the element especially concerned in growth might predominate. In the case of calcium, the combined fraction is relatively abundant when the tissues are poor in calcium and vice versa. Moreover, combined calcium shows an unexpected constancy in terms of dry weight, and it might therefore be concluded on both these grounds that it plays some important part in growth (dry weight production). Against this view is the fact that combined calcium is certainly far from being chemically homogeneous, and includes insoluble crystalline products such as the oxalate which are usually regarded as excretory. The work of Kostytschew and Berg suggests that it may be even more heterogeneous than is usually supposed. We are at present investigating the nature of the combined calcium and it will be best to postpone further discussion concerning its composition and its function until more data are available. Combined magnesium is also of unknown composition and function.

For both potassium and calcium (omitting combined calcium) the partition indices were positively correlated with the weight of the element per 100 gm. dry matter, suggesting that the sap-soluble fractions of these elements are concerned in growth and that the adsorbed fractions are luxury consumed. The suggestion is strengthened by the absence of adsorbed potassium from the old leaves at low levels of potassium supply. But here again it would seem that this obvious conclusion may not be the correct one, for it would appear that the partition of potassium and calcium between sap-soluble and adsorbed fractions is determined by exchange. It is impossible to say what fraction plays the more important role in growth. Possibly the stability of protoplasm is determined by the distribution of the bases between the components in somewhat the same way that bases may control the stability of a clay.

## VI. SUMMARY

1. The bases potassium, calcium, and magnesium have been fractionated into sap-soluble and sap-insoluble forms. For potassium the sap-insoluble form appears to be homogeneous since it is completely soluble in hot water. For calcium and magnesium the sap-insoluble fractions are only partially soluble in hot water, and they have therefore been separated into water-soluble and water-insoluble fractions. The sap-insoluble, hot-water-soluble fraction has been termed *adsorbed*, and the hot-water-insoluble fraction *combined*.

2. It is concluded for potassium and calcium that the partition between the sap-soluble and adsorbed forms is determined to a large extent by exchange.

3. Combined calcium shows a striking constancy per 100 gm. dry matter, and is but little affected by the richness of the tissue in total calcium, by the level of potassium supply or by the age of the leaf. Its chemical constitution and its function are still obscure.

4. The results for magnesium, though rather confused, tend to resemble those for calcium.

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# The Morphology, Cytology, and Sexuality of the Homothallic *Rhizopus sexualis* (Smith) Callen

BY

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With forty Figures in the Text

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## INTRODUCTION

THE fungus forming the subject of this study was originally isolated by Mrs. M. N. Kidd in the Botany School, Cambridge, England, from a rotting strawberry. Through the courtesy of Professor Brooks, a culture of this fungus was received by the Department of Mycology, University of Edinburgh, a few years later, where it has been maintained ever since.

When received it was said to be a homothallic *Rhizopus*, greatly resembling *R. nigricans*. It was never identified until Smith (1939) described it as a new species, *Mucor sexualis*. He used a culture also obtained through Professor Brooks, but gives practically no details of the fungus, apart from the technical descriptions.

Preliminary observations had shown that this fungus belongs to the genus *Rhizopus*. It is therefore proposed to give a more detailed account of its behaviour.

## TAXONOMY

There has apparently been only one homothallic species of *Rhizopus*, that described by Raciborski (1900) as *R. artocarp*i on the male inflorescence of

*Artocarpus incisa* from Java. In his attempts to grow it in pure culture, he obtained zygosporos in only one culture out of many, after one week's growth, and he admits contamination of the culture. From the measurements he gives and his description of the obtuse angle (*einen flachen Bogen*), between the zygosporos and their suspensors, it seems highly probable that he was dealing either with a mixed isolation of the (+)ve and (—)ve races of *R. nigricans*, or, what is more probable, that he had isolated a (—)ve race, which in that one culture had become contaminated with the (+)ve race in addition to the Hyphomycete he mentions; the size of the suspensors and their shape seems to bear this out. The length of time taken by the zygosporos to make their appearance is also typical of *R. nigricans*, and quite unlike any of the homothallic Mucorineae. Zycha (1935) believed this fungus merely to be a homothallic race of *R. nigricans*.

Sartory and Sydow (1913) isolated *R. artocarpi* from the male inflorescence of *Artocarpus integrifolia*, and found that their culture appeared to be identical with that of Raciborski. They only described the sporangia, and did not even so much as mention that zygosporos occurred. There is no evidence to lead one to believe that their fungus was homothallic.

Naumov (1916) was the next to describe a homothallic species of *Rhizopus*, isolated from *Ficus Carica* obtained in the Caucasus. He believed his fungus to be identical with that of Raciborski, though regretting that Raciborski had not described the method of zygosporos formation. In Naumov's opinion this was an essential point, as it constituted the sole means of distinguishing *R. nigricans*, which is heterothallic, and his own fungus, which is equally definitely homothallic.

Kniep (1928) included *R. artocarpi* in his list of homothallic species, attributing the name to Raciborski, but in brackets 'Naumov 1916', probably referring to the fact that the previous descriptions lacked convincing evidence that the species is homothallic.

*R. artocarpi* appears to have been further studied by several Americans, including Harter, Weimer, and Lauritzen (1921), and Weimer and Harter (1923), but they were only concerned with its pathology and do not mention zygosporos or their formation.

A comparison of Naumov's *R. artocarpi* and *R. sexualis* shows many similarities. The figures of the zygosporos given by Naumov (1916) could be those of *R. sexualis*, and his measurements are within the limits of those given in this paper. Altogether the description of the sexual phase of Naumov's fungus agrees fully with that of the present species, but scarcely at all with that of Raciborski. His description of the sporangial reproduction and his measurements do not however, agree with the present species, and rather doubtfully with those of Sartory and Sydow.

It is quite possible that Naumov was dealing with the species under review, but as his fungus appears to differ considerably from that of Raciborski there is no justification for calling it *Rhizopus artocarpi*. The name therefore given

by Smith (1939) stands. The present species is certainly a *Rhizopus* as it forms stolons, at the nodes of which rhizoids and bunches of sporangiophores are produced. Further, the sporangia have distinct apophyses, which clearly distinguishes it from the genus *Mucor*. This fungus must therefore now be called: *Rhizopus sexualis* (Smith) comb. nov. *R. sexualis* is closely allied to *R. nigricans*, though by no means identical with it.

#### MICROTECHNIQUE AND CULTURAL METHODS

It was essential for a close study of the morphology of the fungus, and particularly for the study of the nuclei, to examine the fungus repeatedly under high magnification without contamination. The agar film technique of Kniep as modified by Sass (1929) and Noble (1937) was found to be ideal, and particularly suited to this homothallic *Rhizopus* owing to the scanty production of aerial mycelium. Pure cultures could be maintained with ease for three weeks in such cells without contamination.

The most satisfactory medium was found to be that used by Noble (1937), as it gives a clear evenly staining preparation even when a thicker film is used. The fixing fluid as recommended by Sass (1929), and used by Noble, though satisfactory for the film, was quite unsuitable for the mycelium, as the tips of the hyphae burst immediately the film was immersed in the fluid. In most cases the suspensors, the progametangia, and the zygospores burst, causing extrusion of the protoplasm. After numerous experiments Karpechenko's modification of Navashin's fluid (Rawlins, 1933) was used, as it caused practically no bursting of the hyphae, fixed the film well, and caused no apparent plasmolysis. Fixing for 18 hours and washing for 18–24 hours was found to give satisfactory results.

With this fixative, iron-alum-haematoxylin was most unsatisfactory, and Breinl's triple stain did not give sufficient contrast. A combination of aqueous gentian violet (with a few drops of commercial anilin oil added), iodine, and erythrosine was found to be ideal, as it gave the whole preparation a rose-pink colour, except the nuclei, which stained a deep purple. The successful staining with gentian violet appears to rest largely on the presence of an impurity in the commercial anilin oil used. Great care had to be exercised in using the iodine, as, if it was allowed to act too long, spherical deposits were found on the inside wall, making the preparation useless for further study.

For the other cultural work, a  $2\frac{1}{2}$  per cent. malt extract agar was used, sometimes with the addition of  $2\frac{1}{2}$  per cent. sucrose. When using *Sporodinia grandis*, some rice starch was introduced into one-half of the Petri dish before the medium solidified, but this did not appear to have any beneficial effect.

Material for microtome sectioning was fixed in Fleming's weaker solution, though Navashin's solution as modified by Karpechenko was also used. The former was more successful, though no amount of treatment with hydrogen peroxide would remove the excessive blackening of the maturing zygospores. Colson (1938) met the same difficulty, though in the present case it appears to

be due to the retention of the osmic acid by the oil reserves, as the suspensors stained normally.

The cultures have all been kept in the dark at a temperature of from 18 to 20° C., except during the height of the summer in Würzburg, Bavaria, when the temperature rose to nearly 30° C., though it did not appear to affect them adversely.

#### SOURCES OF MATERIAL

The fungi used in the hybridization experiments were obtained from the following sources: *Rhizopus nigricans* Ehrenb., (+) and (–)ve; *Mucor hiemalis* Wehm., (+) and (–)ve; *M. Ramanianus* Möl.; *Absidia glauca* Hag. (+) and (–)ve; *Zygorhynchus Moelleri* Vuill.; all from the collection maintained in the Department of Mycology, University of Edinburgh. *Mucor hiemalis* Wehm., (+) and (–)ve; *M. mucedo* Fres., (+) and (–)ve; *Absidia glauca* Hag., (+) and (–)ve; *A. cylindrospora* Hag., (+) and (–)ve; *Phycomyces nitens* (Kz) v. Tiegh. et Le Mon., (+) and (–)ve; all from the collection maintained in the Botanisches Institut der Universität, Würzburg, through the courtesy of Professor Dr. Hans Burgeff. *Rhizopus Oryzae* Went et Pr. Geerl.; *Rh. tonkinensis* Vuill., *Rh. japonicus* Vuill., *Absidia coerulea* Bain., *A. Regnieri* (Luc. et Cost.) Lend., *A. capillata* v. Tiegh., *Circinella minor* Lend., *C. spinosa* v. Tiegh. et Le Mon., *Sporodinia grandis* Link., all from the National Type Collection, Lister Institute, London, were supplied through the courtesy of Dr. St. John Brooks. *Mucor albo-ater* Naum. and *Zygorhynchus Vuillemini* Namysl. were obtained from Dr. Marie Waterston (née Campbell).

#### THE MYCELIUM

In all cultures the mycelium that first appears is a submerged type, closely adpressed to the medium, or actually embedded in it. It grows rapidly and is hyaline in appearance. When this mycelium is some forty-eight hours old a superficial type makes its appearance in the centre of the inoculum and spreads out radially.

With increasing age, long stolons are developed, which are so characteristic of the genus *Rhizopus*. They are strong, thick, rapidly developing hyphae which grow beyond the edge of the submerged mycelium, giving forth numerous 'rhizoids' where they come into contact with the medium. These 'rhizoids' remain hyaline and develop into the submerged type of mycelium. For some reason not yet understood the stolons may branch in mid-air, producing from four to six branches, which then grow forward and take root in the medium.

As the mycelium develops, the stolons become fewer in number, their development apparently being arrested at an early stage, and by the end of the fourth day the submerged mycelium is giving rise to short stolons only, which now assume a sexual function, in other words they become zygothores. The stolons are therefore homologous with the zygothores.

The superficial mycelium is light silvery grey in colour, but later loses its lustre, and ultimately assumes a light buff colour.

According to Burgeff (1924), *Rhizopus nigricans* produces 'Fanghyphen', which might be translated as 'trapping hyphae'. They are produced in the normal course of growth before copulation takes place and are thin much branched hyphae arising from the ageing mycelium. *R. sexualis* does not normally produce these trapping hyphae until the culture is several days old, and the aerial mycelium has developed, and then generally only if the culture is in a sufficiently saturated atmosphere. As the cultures become older the new zygothores are very much thinner, and the zygothores correspondingly very much smaller. The zygothores branch excessively, and show a great similarity to the trapping hyphae. It appears highly probable that in this homothallic species the trapping hyphae and the zygothores are homologous. This suggests that trapping hyphae in *R. nigricans* are really only zygothores. In *Absidia*, according to Burgeff (1924), the trapping hyphae are suppressed when sexually similar mycelia are plated together, which suggests that structures comparable to zygothores might be found in other heterothallic species.

In *R. sexualis* very fine hyphae may also be produced by the older stolons, zygothores, and suspensors, if the atmosphere is sufficiently saturated. Their production may be stimulated in young cultures by the presence of other fungi, but generally only in those parts of the homothallic mycelium in contact with the other fungus. These very fine hyphae are produced by the stolons only when they have started to collapse, and this is true also of the zygothores, though not of the suspensors. Further, hyphae in the air near the zygothore are induced to produce these fine hyphae as well, and, with those of the suspensors, help to envelop the zygothore and impart a fur-like appearance to it.

Chlamydospores, as defined by Zycha (1935), are not produced by *R. sexualis*, though present in other *Rhizopus* species. Giant cells are, however, produced as the edge of the mycelium reaches the limits of the nutrient medium, when the tips of the hyphae become very much swollen and distorted, and filled with a dense mass of protoplasm and nuclei. In addition to the giant cells another type of gemma is produced, which might be called 'Reservestoffblasen', but which Zycha (1935) termed 'Dauergemmen'. They are small spherical swellings developed at short intervals along the main growing or trunk hyphae, as outgrowths of the wall, and are either terminal on short branches, or sessile. When fully mature they are dark green in colour, and a cross wall is laid down, cutting off the spherical body from the rest of the mycelium. Although the term 'Reservestoffblasen' has been used, it does not mean that they are identical with the swellings found in *Phycomyces* (Burgeff, 1924). In the latter they are swollen or distorted portions of the hyphae, whereas in *R. sexualis* we are dealing with an actual outgrowth of the hyphal walls.



There is a very marked protoplasmic flow in this species, both to the growing tips of the mycelium and to the developing reproductive organs; reversal of the flow has also been observed, even away from the reproductive organs.

#### ZYGOSPORE FORMATION

When the mycelium is some eighteen hours old, strong rapidly growing hyphae rise into the air from any branch of the mycelium excepting trunk hyphae; these aerial hyphae develop rapidly as there is a constant streaming of protoplasm to them. It is noteworthy that up to this point the mycelium has developed no sporangia. The first of these aerial hyphae to be produced become stolons. The later ones, formed farther from the centre of the culture, are much shorter and produce a lateral branch at least one-third of the way up. Burgeff stated verbally and also in his paper (1924) that the terminal portion of the aerial hyphae cease growing in all the homothallic species known to him, and that cessation of growth is a necessary factor for the production of the lateral branch. It has become increasingly evident in *R. sexualis*, however, that this is not necessarily the case. The lateral grows rapidly, and in a short time curves towards the less rapidly growing tip of the terminal; these two constitute the zygophores. With the realization that the aerial hyphae are really the beginnings of the zygophores, comes confirmation of Burgeff's statement (1924, p. 79), that the sexual phase is initiated before the asexual.

Measurements of the zygophores show the lateral to be always the thicker, but that formed from the terminal portion of the aerial hypha does not decrease in size when growth continues more rapidly after the lateral has been produced. The measurements for the base of the aerial hypha and the terminal portion are identical, being 11–18  $\mu$  in diameter, whereas for the lateral they are 14–22  $\mu$ . If, however, the aerial hypha produces a lateral close to its own base where it arises from the medium, then they both have the same diameter, namely that of the base of the terminal, 11–18  $\mu$ .

In no case have swellings been observed on the zygophores until they have come into contact, which leads to the conclusion that the progametangia are formed through contact stimulus. In the few cases where swellings have been observed without apparent contact (always in fixed preparations), the zygospores have been pulled apart during the fixing. The view that contact stimulus is responsible is supported by the fact that apparently compatible zygophores, that is a terminal and its lateral, may curve towards each other, but do not develop gametangia unless they come into contact.

The progametangia are essentially equal in size, and if one happens to be larger at the beginning, as is often the case, the other develops more rapidly later. The actual gametangia cut off are, however, in almost all cases unequal, the slightly larger gametangium being cut off from the progametangium on the lateral, i.e. thicker zygophore. The aerial hyphae show guttation before the lateral is produced, and the zygophores soon after progametangium

formation. One single drop appears on the zygosporangium when the two gametangia have completely fused.

The zygosporangium takes some twelve hours to develop fully from the time of contact of the zygomorphs. They vary greatly in size according to the age of the culture and are  $54\text{--}172.5 \times 70.5\text{--}220\ \mu$  (100 measurements) (Smith  $60\text{--}180\ \mu$  in diam.) in size, the first measurement being taken across the zygosporangium between the suspensors, and the second at right angles to that. The suspensors measure  $40\text{--}110 \times 48\text{--}128\ \mu$  in size, taken across the suspensor from zygomorph to zygosporangium, and at right angles to that respectively.

During development the suspensors are hyaline, and it is the young zygosporangium that first shows pigment development. As it reaches maturity, however, the suspensors gradually assume a light brown colour, which then spreads down the zygomorphs, but is much less intense there. It is not formed in the mycelium buried in the medium, as Köhler (1935) found was the case in *Mucor mucedo*. Crystals are formed in the suspensors, a character common to the genus *Rhizopus*.

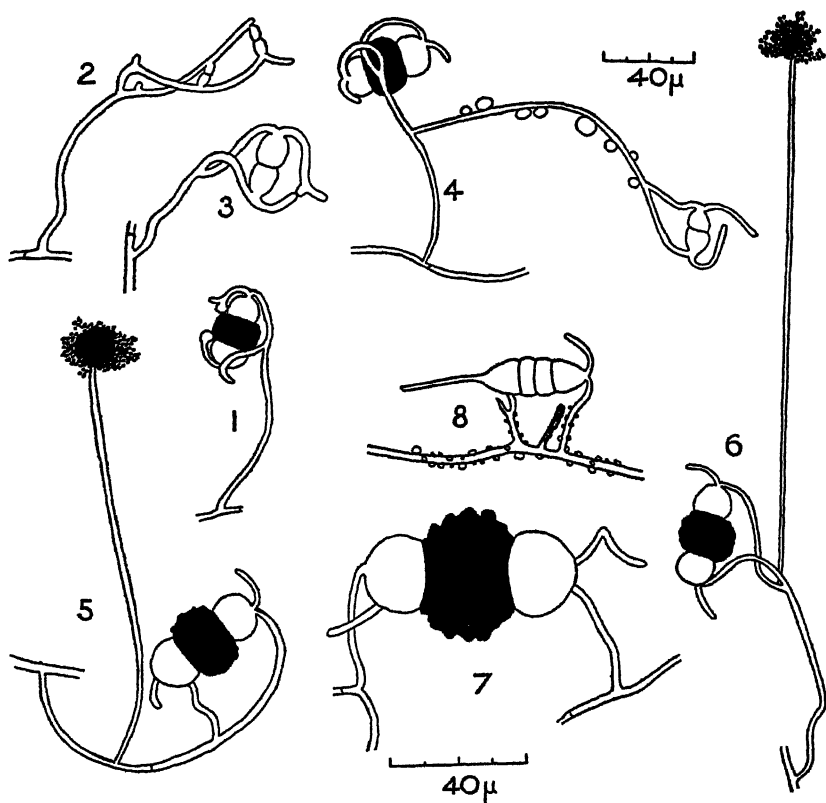
Although it is usual for only one zygosporangium to be produced by the zygomorphs, the ends of these zygomorphs may continue their growth and form yet another zygosporangium, ladderwise. This second one is often unable to develop fully as the rapid growth of the first may cause the pulling apart of the progametangia of the second one (Fig. 1). This production of zygosporangia ladderwise (Figs. 2–3) in homothallic species is proof that the terminal hypha does not completely stop its growth on production of the lateral, but merely slows down, and that it can take up its rapid growth later. The nearest approach to this type of zygosporangium formation has been observed in *Absidia spinosa* by Burgeff (1924) and Nielsen (1927), where one zygomorph was found to be capable of producing two zygosporangia by contact with two different laterals, though one of them is nearly always aborted, due to insufficient nutriment as both authors suggest.

The zygomorphs, however, may not come to zygosporangium formation at first, in which case they produce laterals and these again laterals, which in various combinations may give rise to as many as three or four zygosporangia on the original pair of zygomorphs. If there is no zygosporangium production, the zygomorphs either wither or continue to grow rapidly and become long stolons.

After several days' growth a number of variations can be observed. One of the most common is that the base of the aerial hypha gives rise to a lateral (second lateral) which continues the reproductive phase and does not develop into a stolon. This lateral in its turn gives rise to a lateral branch, the two constituting a new pair of zygomorphs which produce a mature zygosporangium (Fig. 4) or, apparently if insufficient nutriment is present, the third lateral does not develop, and the second lateral produces a sporangium at its apex (Fig. 5). The sporangiophore may be either long or short, but is of the auxiliary sporangial type.

Other variations include that two unbranched aerial hyphae (zygomorphs)

may react together and produce a mature zygospore (Fig. 7). They are of different lengths and ages, and sometimes one of them may have a minute withered lateral. It may also happen that the terminals of two different sets of zygophores copulate. In other words, there is no hard-and-fast rule in order to obtain a sexual reaction.



FIGS. 1-8. *R. sexualis*, types of zygospore and sporangium formation. For further explanation see text. The scale beside Fig. 7 applies to that one only.

On a highly unfavourable medium the stolons are capable of taking up the sexual phase, giving rise to a lateral near the tip, which reacts with the terminal portion to give a mature zygospore. The stolons are incapable of reacting sexually except at the tip, but are capable of producing laterals in the sexually inactive region, which can react together to form mature zygospores (Fig. 8).

Summarizing therefore, it would appear that any young hypha is capable of reacting sexually if sufficient nutriment is present. This follows as a natural corollary to the statement already made (p. 796) that the sexual phase precedes the asexual. That the sexual phase does precede the asexual, even in the heterothallic fungi has been demonstrated for *Phycomyces nitens* by

Burgeff (1924), who stated that the change from vegetative to sexual reproduction takes place much nearer to the growing-tips of the mycelium than the change from sexual to asexual reproduction.

During attempts to secure germination of the zygospores, suspensor regeneration was obtained when four-months-old zygospores were sown on damp sterile plates in a saturated atmosphere of water vapour. Regeneration of single suspensors has been obtained with zygospores over one year old when sown on malt extract agar, and even freezing for a month does not hinder regeneration. As the sporangiospores of this species are not viable beyond four months, it is relatively easy to trace the developing mycelium to the suspensor in the case of old (i.e. over six months) zygospores.

In all cases where regeneration has been obtained, only the true homothallic mycelium has been developed. According to a verbal communication from Burgeff, he himself has been able to isolate only homothallic mycelia from the suspensors of true homothallic species. Satina and Blakeslee (1930) mention regeneration of isolated progametangia of *Dicranophora* '—which may be published later—' which supports the facts above, that the plasma of the progametangium is undifferentiated sexually.

A remarkable variation is noticeable amongst these regeneration isolations. Some have the typical scanty aerial mycelium with stolons, but others develop a varying quantity of aerial mycelium, which at first is white in colour though later buff, and in the extreme cases almost obscures the zygospores produced beneath it. When subcultured, each isolation retains its own special characteristics.

This apparent mutation suggested the possibility of other changes. With a view to testing this, each isolation was plated in triplicate against the Würzburg strain of *Mucor hiemalis* (—)ve, with the original *R. sexualis* as control. *R. sexualis* gives perfect hybrid zygospores with *Mucor hiemalis* (—)ve, as will be shown later (p. 809). It was found that those isolations which had shown a scanty aerial mycelium gave a response equal to that of the control. Those showing a medium development of aerial mycelium gave a distinctly poor response, and those which had so much aerial mycelium that the zygospores could scarcely be seen with the naked eye gave no response at all. In other words, the amount of aerial mycelium present and response to hybridization are linked characters in this species.

Another mutation that appeared in one culture was characterized by the secretion of guttation water of a clear deep crimson hue when seen by transmitted light or black by reflected light. In subculture the mycelium retained this character for several generations, when the culture was unfortunately lost during transit on the Continent. As the same batch of medium was used for all subcultures, the medium cannot have been responsible for this change.

#### SPORANGIAL PRODUCTION

As has already been seen, the zygophores are produced within twenty-four hours of inoculation, but it is only after forty-eight hours that the sporangia

appear. They are at first exclusively formed in those portions of the mycelium in which there is a slowing down or checking of the growth, and only later are they formed in the sparsely covered areas. It can clearly be seen therefore that the sexual phase is initiated before the asexual, as has already been pointed out (p. 796).

The first sporangia to appear are of the type called 'Zwergsporangien' by Burgeff (1924), or as he now prefers to call them, 'Nebensporangien' (see Köhler, 1935), in English 'auxiliary sporangia'. They have been described by Burgeff in *Phycomyces nitens*, and by Köhler in *Mucor mucedo*. In appearance these auxiliary sporangia are merely miniatures of the normal ones, except that they have no rhizoids and the sporangial wall is very much thinner and diffuent, which is well illustrated by Smith (1939). They are produced from the lateral branches of the mycelium and occur singly, though on occasion branched sporangia have been observed.

The normal sporangia are produced by the stolons, but only under certain conditions. If a stolon touches the medium where it is covered with the submerged type of mycelium, then one or more sporangia are formed at the point of contact, and rhizoids are produced which soon develop a dark brown colour. It is therefore only where there is a checking of growth that they are produced, and their absence in Smith's cultures must have been due to the almost total suppression of stolon production through growing the fungus on a highly unfavourable medium. In young cultures the stolons root beyond the edge of the submerged mycelium, and consequently no sporangia are formed. If the stolon does not reach beyond the edge of the mycelium it produces from one to five sporangia (generally one) and ramifies itself amongst the submerged mycelium. On the other hand, if the stolon touches the glass rhizoids are produced, generally without sporangia. This is in direct contrast with *R. nigricans* where bunches of sporangia are usually formed at all points where the stolons touch the glass.

In the most recent monographic treatment of the genus, Zycha (1935) states that in the genus *Rhizopus* as a whole, in any species the only fairly constant character, morphologically, appeared to be the height of the sporangiophores and the size of the sporangia and the spores. In *R. sexualis*, however, the sporangiophore length varies considerably, being anything from 30–192  $\mu$  in height. This is due to the fact that there is no sharp demarcation between the normal and auxiliary sporangia. The sporangia also show a wide range in size, 24–253  $\mu$  (Smith 40–85  $\mu$ ) in diameter, varying more or less with the length of the sporangiophores. The spores themselves are more constant in size, however, and even those produced from the auxiliary sporangia practically fall within the limits of the normal range. The normal spores measure 6–17  $\mu$  (average of 100, 10  $\mu$ ) (Smith 8–18  $\mu$  in long axis) and those of the auxiliary sporangia 9–18.5  $\mu$  (average of 50, 12  $\mu$ ).

By means of a micro-isolator fourteen monospore isolations were made from auxiliary sporangia, but all failed to germinate.

Attempts were made to influence the production of sporangia and zygo-spores by growing the fungus on acid media down to pH 4, but little difference could be observed. By accident some malt extract agar was allowed to become contaminated by bacteria, and this medium when sterilized, though it had a pH of 6.4, due to the buffer effect of the agar no doubt, had a sickly smell owing to the decomposition of the malt extract. This medium caused a retardation in the growth of the medium, though plentiful sporangia were produced after three days, but only of the auxiliary type as Smith obtained on Czapek's medium. The sexual phase remained in abeyance till the cultures were much older, a reversal of the normal procedure. *R. nigricans* behaved differently on this medium; no sporangia were produced in three days, but very obvious zygophores appeared which curled and twisted into knots instead of growing straight out.

Abnormalities in sporangium production are common in the Mucorineae, and several have been recorded for *R. nigricans* by Vuillemin (1902) and Lendner (1908). Exactly similar ones have been observed in *R. sexualis* which need not therefore be described here.

#### AZYGOSPORE FORMATION

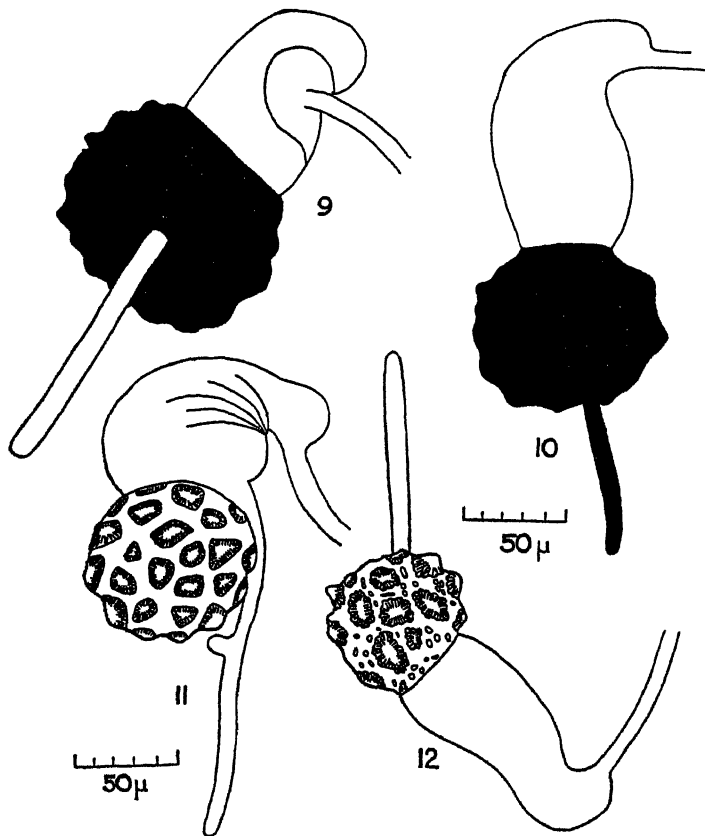
Azygospore production is also fairly frequent in the Mucorineae, and Kniep (1928) was able to give a list of over twenty-five species known to produce them. He placed *R. nigricans* amongst them on the evidence of Zopf (1890) and Namyslowski (1907), the latter describing two types, thigmospores, and ordinary azygospores which he described as rare.

In the present homothallic species azygospore production is actually uncommon, as roughly only ten can be found in a Petri-dish culture (3½ in. diameter) containing some 27,000 zygospores. They may be of two types, simple or double, the double or thigmospore type being much less frequent. A curious structure has been observed on most of the azygospores, which looks like the continuation of the zygophore projecting from one side of the azygospore (Fig. 9). No mention of such a structure can be found in the literature, though Ling-Young (1930) figured a great number of abnormalities. This projection may develop an exosporium at the same time as the azygospore (Fig. 10), though it may only be for part of its length.

On a medium of acid reaction (pH 4.5) there was no apparent increase of azygospore production, contrary to what Kanouse (1923) found in the homothallic *Mucor parvisporus*.

Though the actual number of azygospores produced is very small, the number of pseudophores is very much larger. In hybridization experiments, where azygospore production is greatly stimulated, pseudophore production may be equally stimulated, though this is not always the case. It would appear that some fungi are capable of stimulating the homothallic to cause a far greater number of pseudophores to form azygophores than others, and from an examination of several hundred cultures the writer is forced to the

conclusion that if the mycelium is sufficiently stimulated, pseudophores can develop without the contact stimulus of another hypha. One branched zygophore can in extreme cases produce two or three pseudophores (Fig. 19), which may be terminal or more usually subterminal. In the latter case the terminal portion of the zygophore may continue its growth and give rise to an

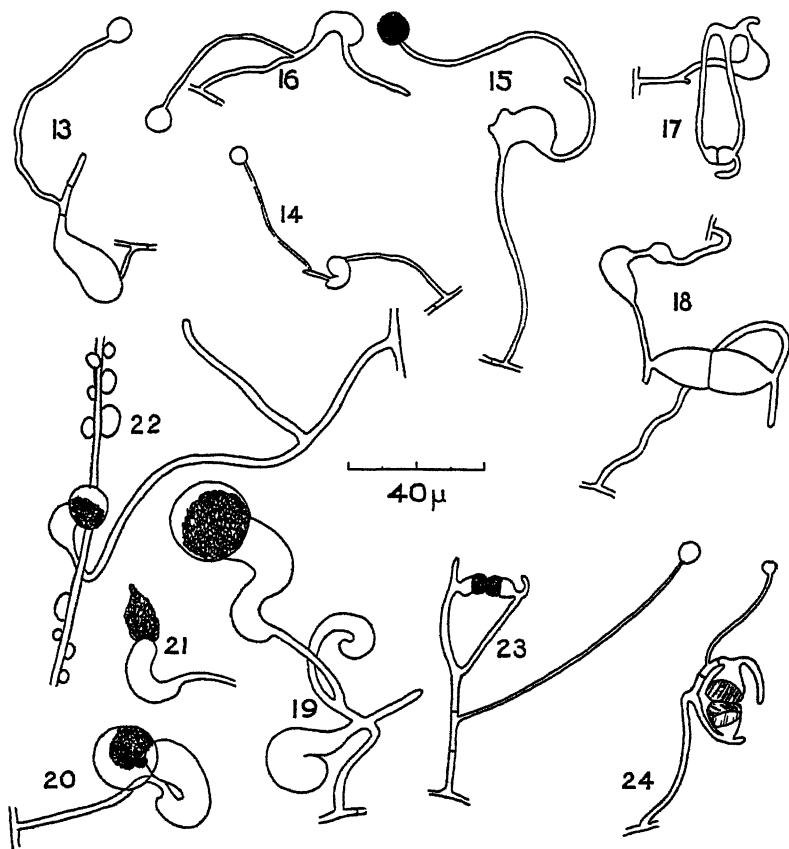


FIGS. 9-12. Azygospores of *R. sexualis* from the following cultures: Figs. 9-11, *R. sexualis*  $\times$  *Mucor hiemalis* (+)ve; Fig. 12, *R. sexualis*  $\times$  *Absidia glauca* (-)ve.

auxiliary sporangium (Figs. 13-16), or even take part in a sexual reaction, either by producing a lateral itself (Fig. 17) or with another zygophore (Fig. 18).

Plasma excretion from the pseudophores has been observed on several occasions. This curious phenomenon has been recorded for *Phycomyces nitens* by Orban (1918) and Burgeff (1924), but is unknown in any other fungus. Orban suggests that it is due to the bursting of the pseudophore, but as Burgeff points out, the pseudophore remains turgescient after this excretion. He finds that the plasma is apparently excreted along with water as it is always

to be found in a large drop of water on top of the pseudophore. In *R. sexualis* this excretion of plasma has been observed on at least seven occasions. On four of these the plasma was lying in a large drop of water (Figs. 19-20) as both Orban and Burgeff have seen it, but on three other occasions the plasma had been found on top of the pseudophore, with no water present (Fig. 21). On



FIGS. 13-24. Figs. 13-16, production of auxiliary sporangia on zygophores associated with pseudophores; Figs. 17-18, zygophores associated with pseudophores taking part in sexual reactions; Figs. 19-22, protoplasmic excretion by pseudophores; Fig. 21, dry excretion of protoplasm; Figs. 23-24, changes from sexual to asexual reproduction.

closer examination of some of the hybridization experiments, this latter 'dry' excretion could be observed relatively frequently. The explanation of the rather puzzling appearance of the tops of the pseudophores only occurred to the writer after much of the hybridization work had been carried out. It would therefore appear that the presence of the drop of water on the pseudophore is only accidental, and that it is not excreted with the plasma as Burgeff suggests. In two of the observed cases where there was a drop of water present it



was obviously guttation water from a neighbouring stolon (Fig. 22). Actually only a very small percentage of the pseudophores produced reach the excretion stage, as most of them are sub-terminal and have the continuation of the zygothore projecting from the tip.

The difference in the exosporium patterns of the azygospores of *R. sexualis* grown in different hybrid cultures (Figs. 11–12) is noteworthy, though at present inexplicable, and requires further study.

#### CYTOLOGY

Examination of the sporangiospores shows that the number of nuclei present varies more or less directly with the size of the spores, and that there is no difference in the number contained in the spores from the auxiliary and normal sporangia. There are from 4 to 20 nuclei in each spore, with either 10 or 12 as the most frequent number. An uneven number can sometimes be observed, but in that case two nuclei are lying side by side as if they were the daughter nuclei of a recently divided nucleus. This rather suggests that the number of nuclei in the spore had increased during the period of swelling, as the counts were made just prior to germination. It was noticeable that the nuclei were unusually large when they numbered only four. This suggestion that a nuclear division takes place in the spore can only be confirmed by an examination of the sporangium, and will be referred to again later.

The zygothores when first formed are like any other hyphae, but additional nuclei are soon brought along by the flow of protoplasm. There is no suggestion of anything other than the ordinary mitotic division occurring in the zygothores. The mere fact that a single regenerated suspensor gives the homothallic mycelium is sufficient proof of this, even although the nuclear divisions cannot be followed.

When the progametangia are formed, the number of nuclei found in them increases rapidly, both by multiplication and migration, though chiefly the latter. At first there is no special aggregation of nuclei, but before the gametangia are cut off the protoplasm becomes much denser on either side of the dividing wall, each mass containing a large number of nuclei evenly distributed. There is no suggestion that they are lying close to the dividing wall, nor that one gametangium is bulging into the other, either at this stage or later. It is at this stage that the mucorine crystals make their appearance, staining deeply with gentian violet.

The gametangia are formed approximately three hours after contact of the zygothores, and contain a large number of deeply staining nuclei which are more clearly defined than those of the mycelium and suspensors. They consist of a deeply staining, spherical, central mass of chromatin, surrounded by a clear non-staining area, and are all of the same size.

Shortly after the gametangia have been formed, the common wall between them breaks down, starting in the central region and working outwards. There is no sign of the protoplasm of one gametangium being extruded into

the other, as observed by Keen (1914) in *Sporodinia*; which suggests that one gametangium is male and the other female. When the wall has broken down the pressure of the suspensors causes the suspensor walls to bulge into the zygote, squeezing its contents into smaller bulk. This is made possible through the great number of loosely reticulated vacuoles of the zygote, which were already present in the gametangia. An association of nuclei in pairs can now be observed, but it is doubtful if there is any fusion at this stage. A number, however, appear to be somewhat larger than their fellows. Some do not take the stain so readily, the central mass of chromatin being ill defined and the clear non-staining area surrounding it less evident; these are presumably nuclei in the process of degeneration.

Some four hours after the formation of the progametangia the contents of the zygosporangium have become remarkably uniform in texture. The nuclei are still as clearly defined as before, with some quite definitely larger than their fellows. These number upwards of twenty, and appear to be increasing in number, though no actual fusions have been observed. Two nuclei may come to be associated, the two deeply staining chromatin masses lying side by side within one clear oval area; they tend rather to lengthen than to retain their spherical shape. This association of nuclei can be first observed when the gametangia are fusing, but it does not appear to be necessary for the two nuclei to originate from different gametangia, as they have been observed associated at points as far removed as possible from the line of junction, and while the common wall was only partially dissolved.

The zygosporangium continues its rapid development, and when it has reached a stage seven hours from progametangium formation, the suspensors stain purple, in contrast with the zygosporangium which still stains a rose pink. This is a sign that the suspensors are past their maximum development and the nuclei are all in the course of disorganization. It is also the stage when the mucorine crystals have reached their highest development; up to ten or more can be counted in a single zygosporangium, as well as several in each suspensor generally close to the wall joining it to the zygosporangium.

When the zygosporangium is examined at later stages, as for example when four days old, the contents are still homogeneous and contained in a thick but transparent membrane fitting closely to the exosporium. On bursting this envelope and staining, the first point to notice is that the contents stain a deep purple, showing that the zygosporangium is in a resting condition. The contents are uniform, there being no differentiation into zones and no oil globules are visible. The nuclei seem to be fewer in number, but that would appear to be due chiefly to the fact that they are lying in pairs. They are large but not so clearly defined as before, and though in pairs there is no suggestion of nuclear fusion. There is no sign of degenerate nuclei. With increasing age there appears to be no further change in the nuclei, and even at four months old the condition is the same as that described above.

Unfortunately it has not so far been possible to bring the zygosporangia to

germination in spite of repeated attempts. Some 25,000, four-month-old zygospores were sown on moist, sterile, circular earthenware plates, and though kept in a saturated atmosphere and in a diffuse light for fourteen days, there was no sign of germination. Zygospores up to the age of nineteen months have been sown on malt agar, after having been allowed to dry up and kept at room temperature, but they also have refused to germinate. The freezing for one month of spores from seven months to two years old has also proved ineffective.

The development of the sporangia is the same as that of *R. nigricans*, which has been fully described by Swingle (1903). When the mass of protoplasm in the sporangium is dividing into little islands, the future spores, from one to four nuclei can be counted in each. It appears that at this stage the nuclei are undergoing active division, so that it is probable that four is not the basic number, and that originally only one or two nuclei were contained in each spore mass. This is contrary to what Swingle found in *R. nigricans*, where there were two to six nuclei in each spore, and there was no division before germination.

Owing to the very small size of the nuclei (only 0.42–0.57  $\mu$  in diameter in the actively growing regions) it is extremely difficult to follow the divisions with any certainty. Moreau (1911) described an amitosis in *R. nigricans*, which appeared to be the normal method of division in the older hyphae, particularly in the columella. This does not appear to occur in *R. sexualis*, however, as divisions can be observed in old vacuolated hyphae and in the columella after the spores have been formed.

The divisions are intranuclear (Fig. 25), and a clear, non-staining area surrounds the dividing chromatin mass, as has been observed to be the case in the Mycetozoa (Wilson and Cadman, 1928; Cadman, 1931). The first sign of the approaching division is the deeper staining of the chromatic mass. The prophase is indicated by an increase in size of this mass, which assumes an irregular oval shape, and stains unevenly. It has unfortunately not been possible to follow the development of the spindle during the prophase, but a lighter staining cone-shaped mass has sometimes been observed on one side of the chromatin; this might be regarded as a portion of the spindle. With the development of the metaphase, the spindle assumes a more or less central position over the mass of chromatin. Centrosomes appear to be absent, though a small deeply staining body can sometimes be observed at one of the apices of the spindle. The spindle itself is pointed at both ends and possesses a uniform structure in which no trace of fibrils can be discerned. The chromatin now divides into two oval masses, each of which can sometimes be seen to be divided into two portions. With the beginning of the anaphase the two masses move to the poles, and in the telophase they are still seen to be connected by the now thread-like spindle. The nuclear membrane elongates as they move apart and begins to constrict in the midline, and ultimately the two daughter nuclei separate completely. A curious structure may often be seen, in what

would be the late anaphase. There are two chromatin masses joined together by the spindle, the whole being crescent-shaped with the chromatin masses at the apices of the horns. In several cases the whole chromatin mass appears to have moved to one pole, the meaning of which is doubtful.

#### HYBRIDIZATION EXPERIMENTS

Quite a number of investigators have succeeded in crossing the (+)ve and (–)ve races of different species and even of different genera. Of particular

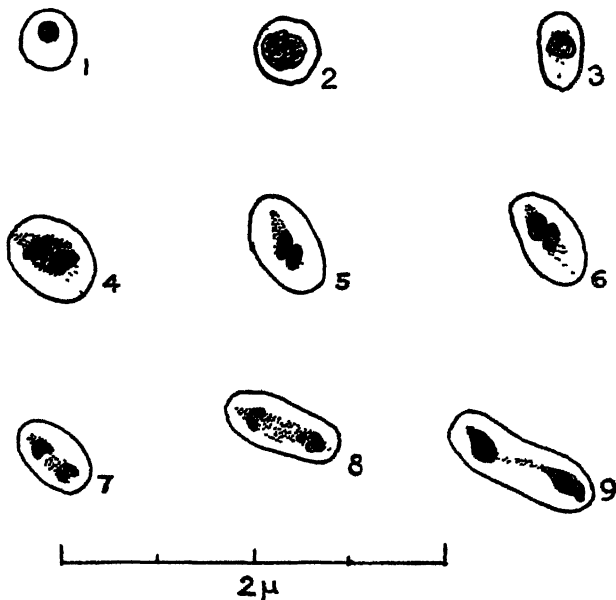


FIG 25. Mitosis in *R. sexualis*: (1) resting stage; (2–3) prophase; (4–6) metaphase; (7–8) anaphase; (9) telophase.

interest, however, are the studies of Blakeslee (1915), which showed that contrasts between homothallic and heterothallic species can give an imperfect sexual reaction. Different homothallic species were found to respond in different ways when contrasted with heterothallic species. Some of them reacted with both the (+)ve and (–)ve races, some did not show any reaction at all, while others showed either a (–)ve tendency and reacted predominantly with the (+)ve races, or had a (+)ve tendency and showed only reactions with (–)ve races.

Burgeff (1924), investigating the hetero-homothallic crosses on a large scale, used the imperfect sexual reaction as an aid in determining the sexuality of the zygomorphs. He employed three different homothallic species and the (+)ve and (–)ve races of two heterothallic species, but in each case only one heterothallic with one homothallic. From his experiments he drew the conclusion

that the predominant (—)ve tendency of the homothallic species used by Blakeslee, was the result of the latter using a particularly active (+)ve race of *Mucor hiemalis*, known as *Mucor V*, as a tester, rather than of a specific (—)ve tendency in the homothallic species themselves.

Nielsen (1927) repeated a number of Burgeff's crosses, with a view to studying the sexuality of the zygomycetes. Satina and Blakeslee (1929, 1930) carried out an extensive programme of crosses, in which they crossed homothallic species with heterothallic and with homothallic species. They reached the same conclusion as Blakeslee, that the heterogamous homothallic species may have either (1) a (—)ve sexual tendency (5 species), or (2) a (+)ve sexual tendency (1 species).

Of great interest are the crosses between homothallic species. Burgeff (1924) crossed *Zygorhynchus exponens* and *Absidia spinosa* and obtained a number of imperfect sexual reactions, but did not pursue this line of investigation further.

Satina and Blakeslee (1930) carried out a number of crosses between various homothallic Mucorineae, chiefly *Zygorhynchus* spp., and found that an imperfect sexual reaction took place between them when one was of a (+)ve tendency and the other of a (—)ve tendency, as they had expected.

In order to determine the tendency of *Rhizopus sexualis*, it was crossed with 25 heterothallic and 3 homothallic races, representing 19 species and 7 genera. The results obtained are shown in the Table. Each cross was quadruplicated, though on occasions as many as six were inoculated for each experiment. Each set of inoculations has been repeated at least four times, in some cases as often as nine, and always the same result has been obtained.

In the first instance *R. sexualis* was contrasted with other species of the same genus. Perfect hybrid zygospores were obtained with *R. nigricans* both (+)ve and (—)ve (Figs. 26–9), though a much larger number were obtained when contrasted with the (+)ve race. In the hybrid the one suspensor is of the large homothallic type. The other does not possess the thin conical shape of the two races of *R. nigricans* used, as is to be expected, but is small and semi-ellipsoidal, with the suspensors of the (+)ve race larger than the (—)ve one. A noticeable feature of the hybrid is the angle at which the suspensors are placed in relation to each other. In the homothallic species they are at an angle of 180°, i.e. opposite, but in the heterothallic used they are generally at an obtuse angle. This is often emphasized in the hybrid to such an extent that they are at right angles to each other. Thus if the smaller suspensor happens to be hidden by the zygospore, it appears as if one were dealing with an azygospore.

Several species of other genera have proved of interest. With *Absidia glauca* (—)ve (Figs. 31–3) the imperfect sexual reaction very often reached gametangium formation, but with the (+)ve race the reaction never passed the early progametangium stage. With *A. cylindrospora* (+)ve (Fig. 30), the chief point of interest is that the exosporium pattern resembles that of the

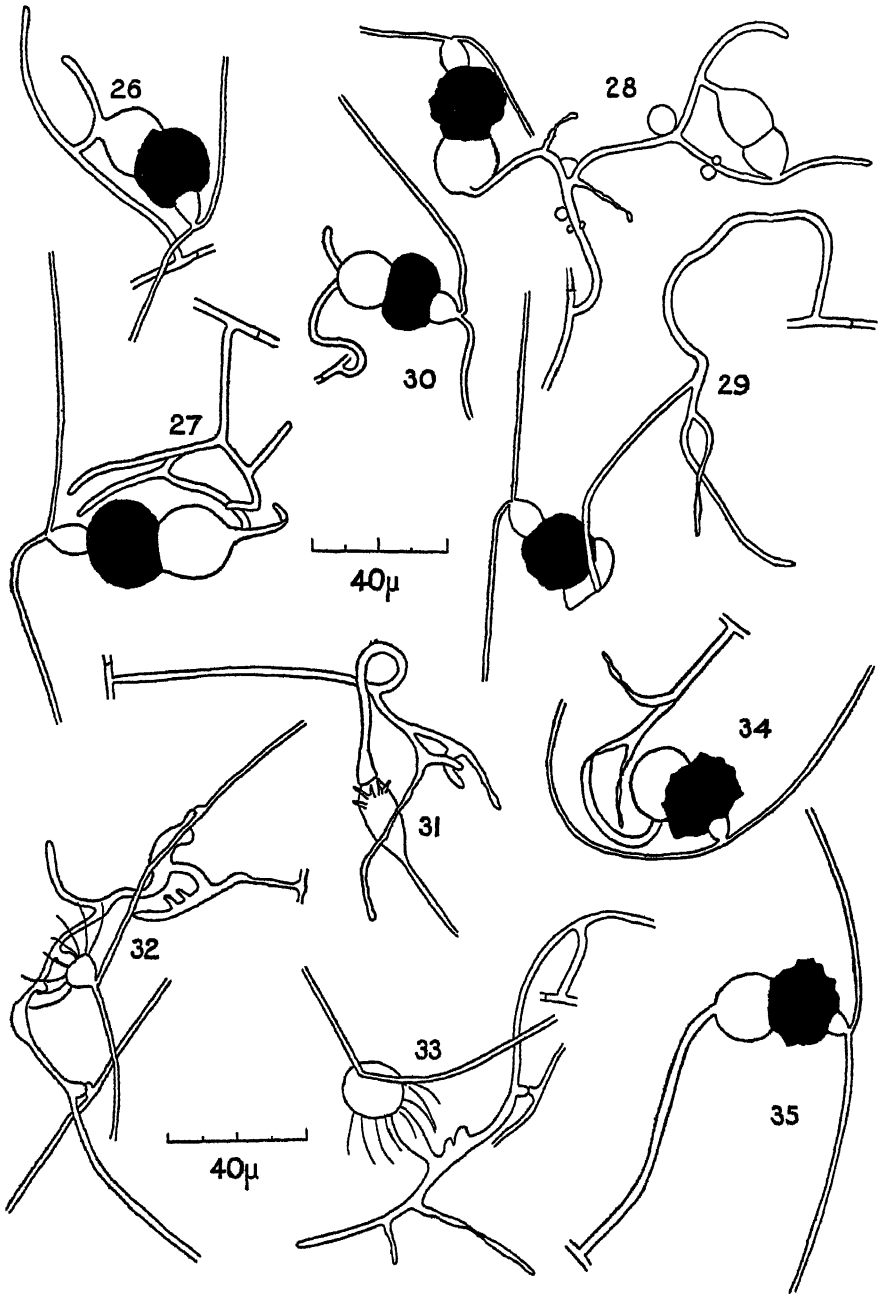
heterothallic parent rather than the homothallic one. Of the *Mucor* spp., *M. hiemalis* (—)ve (Figs. 34–5) proved to be the most interesting, and in this case the exosporium pattern resembled the homothallic parent, though somewhat irregularly so.

Heterothallic and neutral species.	<i>Rhizopus sexualis</i> .	
	Perfect hybrid zygospores.	Imperfect sexual react.
<i>Rhizopus nigricans</i> (+)ve	XXX	X
" " (—)ve	XX	X
<i>R. Oryzae</i>	O	X
<i>R. japonicus</i>	O	O
<i>R. tonkinensis</i>	O	O
<i>Absidia glauca</i> (+)ve	O	X
" " (—)ve	O	XXX
<i>A. cylindrospora</i> (+)ve	O	X
" " (—)ve	X	X
<i>A. coerulea</i>	O	X
<i>A. capillata</i>	O	O
<i>A. Regnieri</i>	O	O
<i>Mucor mucedo</i> (+)ve	O	O
" " (—)ve	O	O
<i>M. hiemalis</i> (+)ve	O	X
" " (—)ve	X	X
<i>M. Ramanius</i>	O	O
<i>M. albo-ater</i>	O	O
<i>Circinella minor</i>	O	O
<i>C. spinosa</i>	O	O
<i>Phycomyces nitens</i> (+)ve	O	O
" " (—)ve	O	O
Homothallic species		
<i>Zygorhynchus Moelleri</i>	O	O
<i>Z. Vuillemini</i>	O	O
<i>Sporodinia grandis</i>	O	O

X slight response; XX moderate response; XXX good response; O no reaction.

Only three homothallic species have been contrasted with *R. sexualis*, and in no case has a sexual reaction of any description been encountered. Blakeslee (1904), Nielsen (1927), and Satina and Blakeslee (1930) have all also failed to obtain any reaction with *Sporodinia grandis*.

To summarize, in those species in which both the (+)ve and (—)ve races were employed, the (—)ve race gave a more vigorous response than the (+)ve one, with the exception of *Rhizopus nigricans* where this was reversed, and *Phycomyces nitens* where there was no response at all. The exceptional behaviour of *R. nigricans* may be accounted for by assuming that we are dealing with an unusually active (+)ve race. This appears to be borne out by the exosporium pattern, where in the cross between homothallic and (+)ve race the heterothallic pattern develops, whereas in the cross between homothallic and (—)ve race the homothallic pattern develops.



FIGS. 26-35. Hybrid reactions. Figs. 26-7, *R. sexualis* × *R. nigricans* (+)ve; Figs. 28-9, *R. sexualis* × *R. nigricans* (-)ve; Fig. 30, *R. sexualis* × *Absidia cylindrospora* (-)ve; Figs. 31-3, *R. sexualis* × *Absidia glauca* (-)ve; Figs. 34-5, *R. sexualis* × *Mucor hiemalis* (-)ve.

The evidence accumulated to date suggests that in *R. sexualis* we are dealing with a homothallic fungus which is predominantly (+)ve in tendency. This is of great interest, as only very few such species are known.

One point ought perhaps to be made clear here. The term 'perfect hybrid zygosporos' is used with the reservation that these zygosporos are not true hybrids, as there is no fusion of nuclei, but are only mixochimeras with an association or partnership of nuclei. Germination of these 'hybrid' zygosporos has not been obtained.

## SEXUALITY

Blakeslee pointed out (1906) that if a sexual reaction could be established between the unequal gametes of a homothallic and the (+)ve and (—)ve races of a heterothallic fungus, the race reacting with the larger female gamete must be considered male, while the race reacting with the smaller male gamete must be considered female. He made use of these reactions later (1913 *a*, 1915), though he was of course assuming that the larger gamete in a heterogamic homothallic was female and the smaller one male. He had also been able to establish (1913 *a*) that it was the lateral zygosporos that gave rise to the larger gametangium. Later work, however, cast some doubts on the sexuality of the lateral gametangium, and finally Satina and Blakeslee (1930) showed that the lateral, and consequently the larger, gametangium could be (—)ve instead of (+)ve in reaction, as in the species *Zygorhynchus heterogamus*.

Burgeff (1924) observed that often the terminal and lateral zygosporos did not come to copulation. They continued their growth instead, and ultimately the lateral gave rise to a lateral of its own. The question which he tried to solve was, how does the second lateral stand sexually in relation to the others? He stated that a sexual substance is manufactured in the tips of the growing hyphae, whether the other partner is present or not, and that this can be transmitted through the substrate or the air.

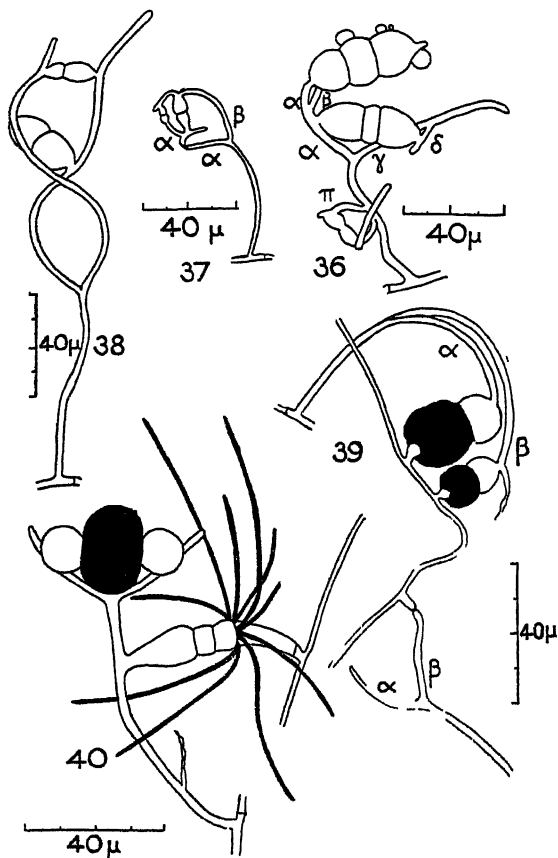
Bearing this in mind, Burgeff turned to the zygosporos of the homothallic species *Absidia spinosa*. He found that the second lateral (i.e. the lateral arising from the first lateral) could copulate either with the first lateral or with the terminal. He therefore postulated that the terminal zygosporos reacts (—)vely, and the first lateral (+)vely, but if they do not come to copulation the first lateral changes to (—)ve (i.e. becomes terminal) and produces a lateral ('second lateral') in its turn, which is then (+)ve. He postulated that the lateral branches can change only once from (+)ve to (—)ve if they do not come into contact with another zygosporos, and that they remain (—)ve.

From a study of a second homothallic species, *Zygorhynchus exponens*, and its sexual reactions with heterothallic species, he was able to draw up a scheme of the sexual zones of the zygosporos.

Nielsen (1927) carried out observations on *Absidia spinosa*, and considered the fact that a single zygosporos can form more than one zygosporos is not consistent with Burgeff's theory. He was of the opinion that the most natural



explanation is that the hyphae contain both sexes, perhaps one sex at first but two at a later stage. He was unable to say whether the terminal hypha turns bisexual, as he had been unable to find cases where there had been a sexual reaction between two terminals.



FIGS. 36-40. Sexual reactions: for explanations see text. Fig. 39, *R. sexualis*  $\times$  *R. nigricans* (+)ve; Fig. 40, *R. sexualis*  $\times$  *Abidia glauca* (-)ve.

Satina and Blakeslee (1930) summarized their work to date and came to the conclusion that the terminal and lateral zygophores in those species which they had investigated were bisexual.

On applying Burgeff's theory to *Rhizopus sexualis* the explanation of the reaction of the zygophores does not appear to be straightforward. In Fig. 36 the first zygospore has been formed between the terminal  $\alpha$  (-ve) and the lateral  $\beta$  (+ve). After its formation  $\alpha$  gave rise to a second lateral  $\delta$  (+ve) which then formed a zygospore with  $\alpha$ .  $\alpha$  has given rise to a third lateral,  $\pi$ , which has branched right at its point of origin, though one must be (+)ve and

the other (—)ve, according to the theory, as there has been a sexual reaction between them.

The chief features of interest presented by Fig. 36 are the production of laterals below the branching  $\alpha\beta$ , each successive one farther down towards the base, the oldest part of the zygophore. This point will be mentioned again later. The lateral  $\pi$  has as yet not been seen to copulate with  $\alpha$ .

In Fig. 37 the first zygospore has been formed between  $\alpha$  and  $\beta$ , which is quite normal according to Burgeff's theory. But the production of the second is certainly not normal, as we have a sexual reaction between  $\beta$  and  $\delta$ , both (+)ve hyphae. The development of this set of zygophores was observed at intervals over a period of ten hours, so that there is no doubt that it is the  $\beta$  hypha which has produced the two zygospores. It was manifestly impossible to determine with any certainty which was the  $\alpha$  hypha and which was the  $\beta$  hypha at the end of the ten hours (Fig. 1). Even if one calls the  $\alpha$  hypha the  $\beta$ , and the  $\beta$  hypha the  $\alpha$ , the case could only be explained by Burgeff's theory by assuming that the zygophore bearing the first zygospore had changed its sex to (—)ve. This is, however, quite impossible in view of the case to be dealt with.

In Fig. 38 is a case where the zygophores  $\alpha$  and  $\beta$  have formed one zygospore and then a second one, ladderwise in relation to the first one. According to Burgeff (verbal communication to the author), this type of zygospore formation is quite unknown in the homothallic Mucorineae. As no laterals have been produced, Burgeff's theory of changing sex does not apply, the zygophores retaining their original sexuality.

In the cross *R. sexualis*  $\times$  *R. nigricans* (+)ve (Fig. 39) a case was observed where the two zygophores  $\alpha$  and  $\beta$  of the homothallic species had both formed zygospores with the same zygophore of *R. nigricans* (+)ve, and in addition the  $\beta$  hypha (+ve) of a second zygophore is in the process of reacting with the same hypha of *R. nigricans* as the previous two. In a second case, Fig. 26, *R. nigricans* (+)ve has formed a perfect zygospore with the  $\beta$  hypha (+)ve of a *R. sexualis* zygophore. It is almost unnecessary to remark that the cultures were not contaminated.

The case figured in Fig. 40, in which there has been an imperfect sexual reaction between *R. sexualis* and *Absidia glauca* (—)ve, is interesting. Here the *Absidia* has reacted directly with the zygophore below the forking  $\alpha\beta$ . According to Burgeff, this is a region which should be either non-reacting or of (—)ve sexuality, as the hypha  $\alpha$  changes its sexuality from (+)ve to (—)ve on the production of the lateral  $\beta$ . Considering the cases of this type, as exemplified by Figs. 36 and 40, we are forced to the conclusion that in *R. sexualis* any part of the zygophore may react sexually, and that there is no passive region.

Nielsen, Blakeslee, and Burgeff had never observed a case where two terminal zygophores had reacted together and formed a zygospore between them. In *R. sexualis*, however, this has been observed on a number of occasions,

either between the unbranched terminal zygophores (Fig. 7), or between the  $\alpha$  hyphae of branched zygophores. Burgeff explained personally that this is quite in keeping with his theory, as the hyphae were of different lengths, and presumably of different age, and consequently of different sex. One must conclude, therefore, that he now assumes that the  $\alpha$  hypha can change its sex before giving rise to a lateral, that it changes its sex when it reaches a certain age or length as a matter of course.

In the light of the cases just dealt with, the theory of changing sex as advanced by Burgeff in 1924 cannot be accepted without modification. Nielsen's explanation (1927), that the zygophores are unisexual first and then bisexual, is not tenable on account of the very nature of the nuclear content of true homothallic species. The only possible explanation that can be drawn is that the zygophores are bisexual the whole time, which confirms the findings of Satina and Blakeslee (1930).

#### DISCUSSION

Since this work was carried out (1936-9), papers by Hartmann and Moewus have come to the writer's notice. Circumstances do not allow the work to be continued in the light of these recent discoveries. The booklet by Hartmann (1939), however, summarizes his own and Moewus' work, as well as that of others, and points the way to an understanding of the behaviour of *R. sexualis*.

It had generally been assumed that in isogamous homothallic species there must be some internal differentiation. The first step was to look for a nuclear fusion. In *R. sexualis* the nuclei in the zygospore were associated in pairs, which possibly fuse on germination, to be followed immediately by the reduction division. It is perhaps worth emphasizing again that it was observed that the two nuclei of a pair do not necessarily come from different gametangia.

The present findings agree with those of Satina and Blakeslee (1929, 1930), that homothallic species are potentially bisexual throughout. It therefore follows that the male and female determining genes only determine which of the two potential sexes is to be dominant. What the action of these genes actually is need not be discussed here, except to state that the one sex masks the other, and that the masked potentiality may still be called forth.

As these homothallic species are potentially bisexual, it follows that not only the suspensors but also the gametangia themselves possess the latent potentiality of the opposite sex.

In 1925 Hartmann described the phenomenon of 'relative sexuality' in *Ectocarpus*, and since then it has been discovered in other algae and in some fungi (Hartmann, 1939). Hartmann defined sexuality as the presence of differences within one sex, differences in degree of the strength of that sex. The work of Blakeslee and his associates has shown that in the dioecious (heterothallic) species of *Mucor* a given race is either (+)ve or (—)ve, or fails

to show any sexual reaction and is then classed as neutral. Some of these neutral races have proved to be either (+)ve or (-)ve, but not of sufficient sexual activity or strength to take part in zygospore formation with the great majority of the races used. According to Satina and Blakeslee (1929, p. 736), 'various grades of sexual vigour are apparent in the different races within a single species'. This is relative sexuality as defined by Hartmann, though Satina and Blakeslee deny the existence of relative sexuality in the heterothallic species of *Mucor*.

It was expected that relative sexuality would occur in *R. sexualis*, and this was the case. In the homothallic species, however, relative sexuality functions in a slightly different way. Moewus (1938) has been able to prove biochemically for *Chlamydomonas* that the sexual substances consist of varying mixtures of cis- and trans-croctindimethylesters, the female substance consisting largely of cis-ester, and the male largely of trans-ester. In the homothallic, assuming a similar sexual substance, one must conceive the zygophore as first secreting the unstable cis-ester, which gradually changes to the stable trans-ester. In doing so the zygophore passes through all grades from a very strongly reacting to a weakly reacting female, then to a weakly reacting, and finally to a strongly reacting male. The lateral zygophore, being younger than the terminal, will contain a larger quantity of the unstable cis-ester, and will therefore have as the dominant sexual tendency a tendency nearer the female end of the scale than the terminal. The best example of relative sexuality in *R. sexualis* is shown in Fig. 39, where the terminal and lateral of the same zygophore of the homothallic have formed perfect zygospores with the same zygophore of *R. nigricans*, (-)ve.

It is evident, therefore, that Burgeff's theory (1924) is basically correct. The modification that he himself proposed (verbally), that the zygophores change their sex automatically without the production of a lateral as they get older, is correct. A further modification of the theory must be made in the light of the work by Moewus (1938) on *Chlamydomonas*, that if there is a sufficient difference in strength between two apparently similar sexes these two will react together. This possibly is the explanation of the cases shown in Figs. 37 and 38. In the latter the  $\beta$  zygophore is long enough to have changed to a weak male after the production of the first zygospore, but the  $\alpha$  zygophore will be a much stronger male. The sudden cessation of gametangium development as shown in Figs. 23 and 24 is probably due to the cis-trans balance being upset through protoplasmic flow, the balance becoming practically the same in each gametangium.

One further point in the work of Moewus (see Hartmann, 1939) is important. In examining the progeny of the cross of the heterothallic *Protosiphon botryoides* he obtained normal male and female races but, in addition, 3 per cent. were homothallic. We know from cytology that such a segregation is caused by an exchange or mutation of genes. It must therefore be assumed that the 3 per cent. homothallic individuals have arisen from just such a

mutation. The male and female determining genes must therefore lie on the same chromosome. In the last reduction division before the formation of the homothallic, therefore, one chromosome has received both sex determining genes, and the other none. The nucleus with no sex-determining genes was actually found to degenerate. This offers an explanation of the sudden appearance of this homothallic *Rhizopus*. Probably Naumov obtained the same mutation. This explanation would also account for the great similarity of *R. sexualis* and *R. nigricans*.

#### SUMMARY

*Rhizopus sexualis* (Smith) comb. nov. is fully described morphologically. The most interesting points have proved to be (1) the production of zygospores ladderwise on one pair of zygothores; (2) the regeneration of single suspensors gives only homothallic mycelium; (3) both auxiliary ('Nebensporangien') and normal sporangia are present, though only the latter produce rhizoids; (4) plasma excretion occurs; (5) two terminal zygothores may react together to produce a zygospore.

The general cytology is described. No chromosomes are differentiated. No fusion of nuclei has been observed. Nuclei associate in pairs from the time of gametangium fusion, though it does not appear that the individuals of a pair must come from different gametangia. This association is still evident in zygospores after several months, practically no nuclei being single. Some of the nuclei entering the zygospore degenerate. Germination of zygospores up to twenty-five months old has not been obtained.

Perfect hybrid zygospores have been obtained when *Rhizopus sexualis* was crossed with *R. nigricans* (+)ve and (–)ve, *Mucor hiemalis* (–)ve, and *Absidia cylindrospora* (–)ve, and imperfect sexual reactions with a number of other species.

*R. sexualis* appears to be of a predominantly (–)ve tendency.

Burgeff's theory of changing sex in homothallic zygothores is rejected, though the modification, as yet unpublished (see p. 813) that he now proposes, that two hyphae must be of different age to react together, is correct.

The findings of Satina and Blakeslee that homothallic zygothores are bisexual is confirmed.

Relative sexuality is discussed in the light of work by Hartmann and Moewus. It is suggested that the fungus has arisen through mutation.

The writer would like to acknowledge his indebtedness to Professor Dr. H. Burgeff for the interest he has taken in this work, and for placing the facilities of the Botanisches Institut der Universität, Würzburg, at his disposal for several months. Special thanks are also due to Professor Dr. E. Münch for similar facilities at the Forstbotanisches Institut, Munich, for a whole year. To Dr. J. Ramsbottom and Mr. I. Mackenzie Lamb of the Natural History Museum,

London, and Mr. George Smith of the School of Tropical Medicine, London, the writer would like to express his thanks for their courtesy. To Dr. Malcolm Wilson particular thanks are due for originally suggesting an investigation of this fungus, and for stimulating criticisms and discussions.

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## NOTE

**A SIMPLE CLASS APPARATUS FOR THE QUANTITATIVE DETERMINATION OF OXYGEN EVOLUTION IN THE PHOTOSYNTHESIS OF *ELODEA CANADENSIS*.**—The use of shoots of *Elodea canadensis* for the demonstration of oxygen evolution in photosynthesis has long been popular in the classroom and laboratory. The detection of dissolved oxygen in the surrounding aqueous medium by chemical methods (Engelmann's blood method, Kolkwitz's reduced indigo-carmin method, &c.) form pretty and conclusive qualitative methods that are simple to perform. The evolution of gaseous oxygen from the cut ends of the shoots can also be readily induced, and the technique of Wilmot (Proc. Roy. Soc. B, xcii. 304, 1921) for the quantitative measurement of photosynthesis by counting the rate of production of uniform bubbles from such cut shoots is now well known and widely used. The method was not new, but by the introduction of several refinements Wilmot eliminated many of the main sources of error in the method and made it capable of very considerable precision.

The simplicity of the method commends it for student experiments. For simple demonstrations the refinements of Wilmot's technique are obviously superfluous, but for more accurate determinations a Wilmot's bubbling cup should be employed. This involves the sealing of the cut ends of shoots into the small bubbling nozzles with hot luting wax, and I have found that to do this satisfactorily requires a degree of skill possessed by relatively few students.

The description which follows is of a simple class apparatus which I have found to yield very satisfactory quantitative results even with first- and second-year students of 'Final' standing. Honours students, with more time at their disposal, obtain results that are all that could be desired. In this method, instead of using the rate of production of uniform bubbles as a measure of the assimilation rate the total quantity of oxygen evolved over a given interval is collected and measured in a microburette. With this arrangement Wilmot's refinements for ensuring uniform bubble size are unnecessary, but his technique in the preparation and use of the experimental solutions is still applicable, and should be strictly followed; for details reference should be made to the original paper.

The essential part of the apparatus (Fig. 1) consists of a glass tube A, about 1 to 1.5 cm. internal diameter, pierced by a number of large holes. The top of this tube is drawn out and sealed to the capillary tube C, which constitutes the microburette. This capillary tube is of 1 mm. internal diameter and is provided with a graduated scale S. To the other end of the microburette two side arms T and M are sealed. The bulb-shaped upper arm T is a gas trap which opens to the air through a rubber tube and pinch-cock. The lower downwardly projecting arm M ends in a length of large-bore rubber tubing R, which is sealed at the lower end by a solid glass rod. A large screw pinch-cock P is fixed on to the wooden stand supporting the whole apparatus, enabling this large-bore tubing to be compressed under careful control.

The shoot of *Elodea* to be used is introduced, cut end uppermost, into the perforated glass tube A. This can best be done by tying a length of thread lightly



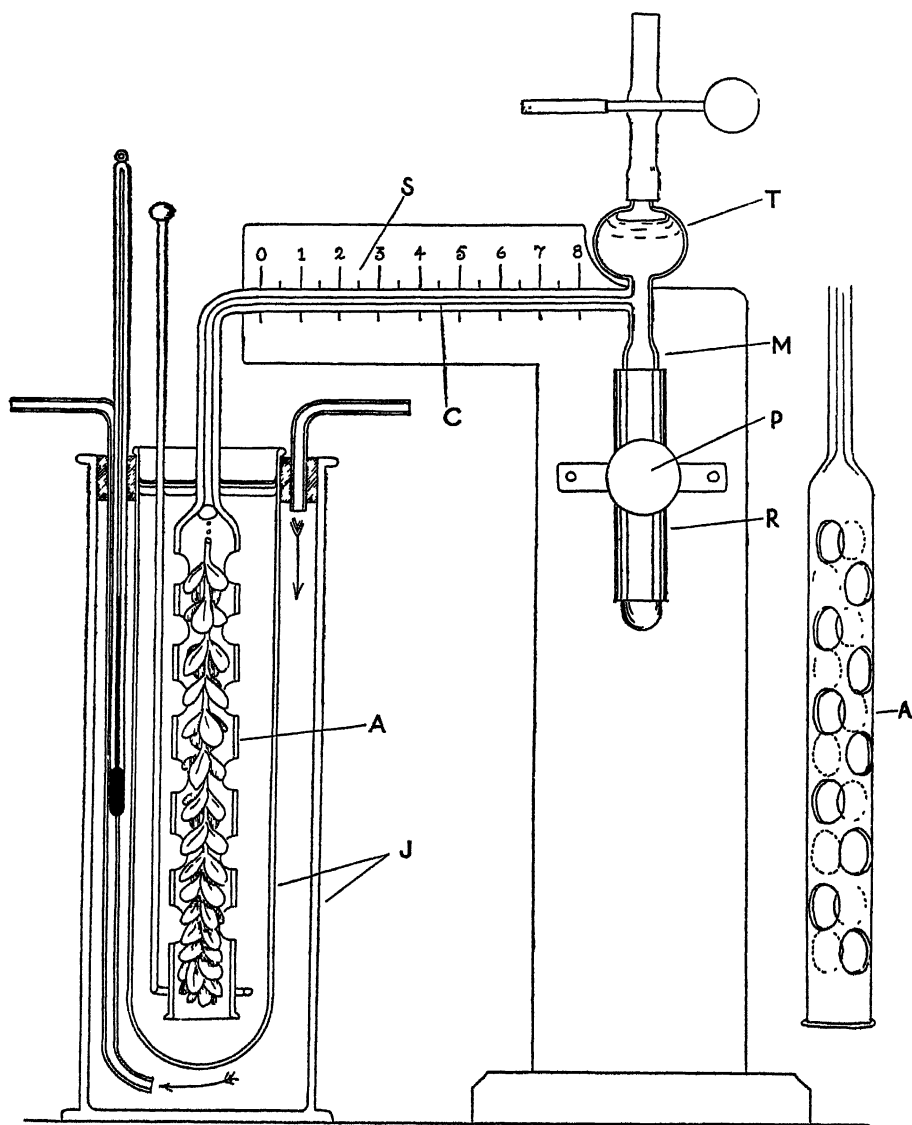


FIG. 1. Diagram of apparatus.

around one of the lower nodes of the shoot, threading it up the tube and through the uppermost hole and then gently pulling the Elodea into position, where it will stay during the subsequent experiment provided the perforated tube is of suitable bore.

The experimental solution is contained in the inner tube of the glass water-jacket J. When the Elodea has been introduced into tube A the gas-collecting apparatus is lifted and the tube A lowered into the experimental solution. By opening the pinch-cock on arm T and applying suction the whole apparatus can be filled with solution.

Illumination of the Elodea is carried out through the water-jacket and the bubbles of gas evolved from the cut end of the Elodea shoot collect as a large bubble at the junction of tube A and the microburette C. After convenient intervals of time, by suitable manipulation of the screw pinch-cock P this gas bubble can be drawn rapidly into the microburette, its length measured, and thence the volume calculated in cubic mm. After measurement further manipulation of the screw-cock P brings the bubble to the end of the capillary where it is released into the gas trap T. In this last adjustment another bubble may be brought into the burette from the top of tube A. If this occurs it is a simple matter to retain it inside the burette while the measured bubble is brought into the gas trap and then subsequently to return it to tube A. The manipulation of bubbles in the capillary tube can be made very delicate by suitable choice of tubing R and screw pinch-cock P. In this way continuous readings of gas evolution over short intervals of time can be obtained.

This apparatus has many uses and can be employed to illustrate the effects of a number of external conditions on the rate of photosynthesis. The effect of light-intensity is perhaps the simplest. Different intensities of light can be produced by varying the distance of the Elodea from the light-source and using the law of inverse squares to determine relative intensities. The effect of  $\text{CO}_2$  supply is readily demonstrated by changing the concentration of solution used. The process of emptying the apparatus and inner tube of the water-jacket of one experimental solution and refilling both with another takes no more than fifteen seconds. Similar technique can be employed for the investigation of the effects of dissolved substances on photosynthesis rates. Again the relationship with temperature can be demonstrated by altering the temperature of the water passing through the water-jacket J. For this an apparatus of the 'geyser' type for delivering a stream of water at various temperatures is required.

To illustrate the type of result which can be obtained with this apparatus the following graphs of typical experimental results have been appended. In Fig. 2 are the results of a three-hour experiment with various light-intensities. The experimental solution was of 2 per cent.  $\text{NaHCO}_3$  saturated with oxygen, and this was kept constantly stirred during the course of the experiment. By this means effects due to gradients of oxygen and  $\text{CO}_2$  were kept at a minimum (see Wilmot). It has been found that a glass loop stirrer round tube A moved up and down by hand was very efficient in bringing about circulation of the solution through the holes in A and round the Elodea shoot. The light-source was a 1,000-watt filament lamp, producing an intensity of 2.5 arbitrary units at a distance from the shoot of 20 cm. Three-minute readings were taken. Light-intensity changes produced immediate adjustments in the rate of photosynthesis to new levels in the first two readings, and the points plotted are for a series of 3-minute readings at each light-intensity, leaving out the initial values during the adjustment.

Fig. 3 shows the results of another three-hour experiment at constant light-intensity and with  $\text{NaHCO}_3$  solution of various concentrations. All these solutions were made up in water saturated with oxygen and the solutions were kept constantly stirred during the experiments. Again adjustments of photosynthesis rates in response to changes in solution concentration were rapid and took place in the first two three-minute readings.

In both these graphs smooth free-hand curves have been drawn through the points. These values are of apparent assimilation and no attempts have been made to

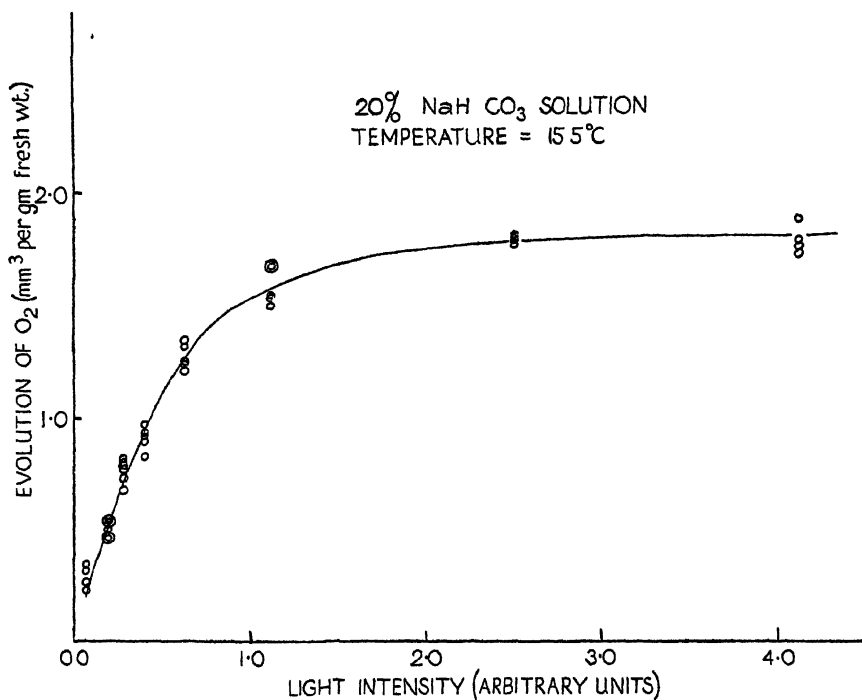


FIG. 2. Results of a typical three-hour experiment on assimilation rate in various light-intensities.

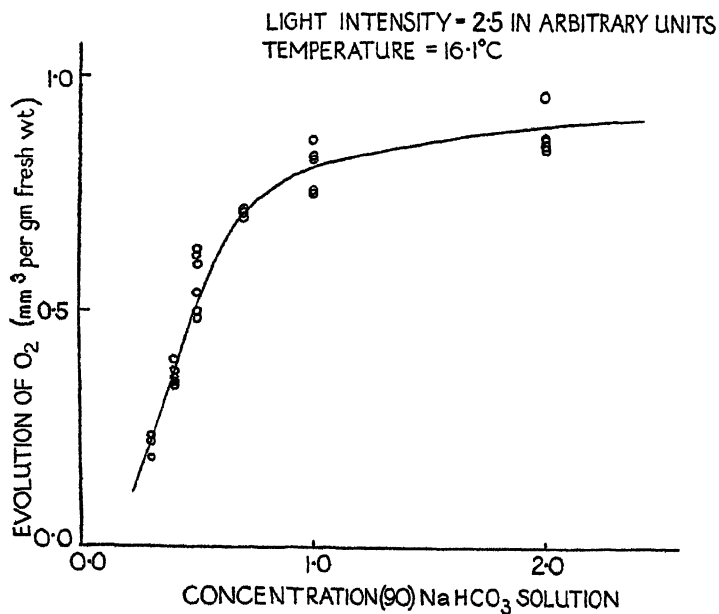


FIG. 3. Results of a typical three-hour experiment on assimilation rate in various concentrations of  $\text{NaHCO}_3$  solution.

correct these for concurrent respiration. The curves show a remarkable approach to those obtained by more elaborate methods.

The value of this simple apparatus is further illustrated in Fig. 4, where the results of temperature change are shown. Assimilation rates were followed in 2 per cent.

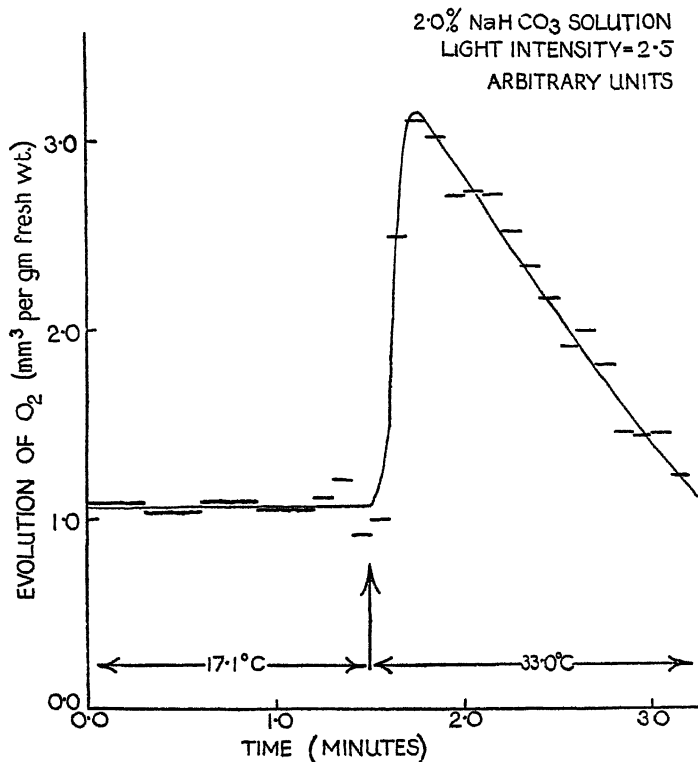


FIG. 4. Results of a short experiment showing the effects of temperature change on the rate of assimilation.

NaHCO<sub>3</sub> solution at a light-intensity of 2.5 arbitrary units at 17.1° C. (tap water). Water at 33° C. from a 'geyser' type electric heater was then passed through the water-jacket and within two minutes the assimilation rate had reached a new high level. The initial lag is that time necessary for the temperature of the experimental solution to reach that of the water-jacket. The subsequent decline of the photosynthesis rate illustrates admirably the deactivation which takes place at this high temperature. A measure of the  $Q_{10} = 1.96$  over this range was found from the peak assimilation value after the transition. This experiment took just over half an hour to complete.

Finally it should be noted that the apparatus can be very easily constructed by anyone with a little experience in glass-blowing, and is sufficiently small and transportable to take its place in large classes with such well-known instruments as the simple potometer, the Darwin porometer, &c.



